Germline and Somatic Neurofibromatosis Type 1 Aberrations in Breast Cancer

A Thesis submitted for the Degree of Doctor of Philosophy

THE UNIVERSITY of ADELAIDE

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Thesis Declaration

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Abstract

Title

Germline and Somatic Neurofibromatosis Type 1 Aberrations in Breast Cancer

Overview

These studies were initiated after seeing a series of women with Neurofibromatosis Type 1 (NF1) and breast cancer (BC) at National Cancer Centre Singapore (NCCS) from 2006 to 2009. Neurofibromatosis type 1 (NF1) is a relatively common tumour predisposition syndrome related to germline aberrations of *NF1*, a tumour suppressor gene. NF1 is usually a clinical diagnosis as individuals with NF1 typically develop multiple neurofibromas which can be cosmetically disfiguring, in addition to other features such as café-au-lait spots, skin tags and Lisch nodules.

These patients under my care had aggressive HER2-positive breast cancers that did not seem to respond to standard systemic therapies as well as in individuals without NF1 syndrome. Individuals with NF1, an autosomal dominant genetic disorder, are known to be at increased risk of developing various tumours, such as malignant peripheral nerve sheath tumour (MPNST), phaeochromocytoma, glioma, and rhabdomyosarcoma. In 2007, the first study which reported an increased risk of breast cancer in women with NF1 was published. Since then, there have been a number of other epidemiological studies with the consistent finding that women with NF1 are have a three- to eight-fold increased risk of breast cancer, especially for women aged less than 50 years. Data on the characteristics of BC in NF1 patients is currently still limited. Our group was the first to discover the higher frequency of HER2-positive, hormone receptor negative and grade 3 breast cancers in women with NF1 compared to breast cancers in women without NF1. We have also performed genomic profiling of these NF1-associated breast cancers.

Over the course of my candidature, large-scale exome or genome sequencing studies led by various groups such as The Cancer Genome Atlas (TCGA), International Cancer Genome Consortium (ICGC) and METABRIC, have revealed somatic *NF1*

aberrations in different sporadic tumours from individuals in the absence of a clinical diagnosis of NF1. These somatic *NF1* alterations appear to be associated with resistance to standard therapy and adverse outcomes, similar to the breast cancers in women with clinical NF1 syndrome. Improved understanding of the implications of *NF1* aberrations is critical for the development of novel therapeutic strategies.

In Asia, women with breast cancer are on average younger than in Western populations, resulting in higer rates of poor prognosis breast cancers in premenopausal women. Since somatic *NF1* mutations in BC are associated with poor prognosis, we also aimed to explore the potential role of *NF1* and neurofibromin in the sporadic BCs from patients without NF1. This included immunohistochemical staining of tissue micrroarrays, and targeted gene sequencing (with *NF1* in the gene panel).

Structure of Thesis and Research Questions

Chapter 1: Systematic Review

This literature review focused on the germline NF1 disorder, the biology of the NF1 gene and neurofibromin, tumours associated with NF1 as well as sporadic tumours harbouring somatic *NF1* aberrations in individuals without NF1 disorder. This review identified an important role of *NF1* in carcinogenesis as well as the challenges of detecting *NF1* aberrations and deficiency or dysfunction of the encoded protein neurofibromin. It also highlights the need to pursue further research, especially in the area of therapeutic strategies for individuals with germline NF1 syndrome and for sporadic tumours with somatic *NF1* aberrations.

Chapter 2: Whole exome sequencing of multiple tumours from an NF1 patient

This paper describes the exome sequencing of BC, MPNST, and neurofibroma from a patient with NF1. Apart from the germline *NF1* mutation, we demonstrated independent somatic *NF1* mutations in all three tumors. Each tumor had a distinct genomic profile with mutually exclusive aberrations in different genes. Although second-hit *NF1* mutation may be critical in tumorigenesis, different additional mutations are required to drive the formation of different tumors.

Chapter 3: Comprehensive case series of BCs in women with NF1 with molecular insights into its aggressive phenotype

The aim was to elucidate the clinical, pathological and molecular characteristics of NF1-associated BCs at National Cancer Centre Singapore. There was a higher frequency of grade 3, oestrogen receptor (ER) negative and human epidermal growth factor receptor 2 (HER2) positive tumours among NF1 patients with inferior overall survival compared to non-NF1 BCs. Immunohistochemical expression of neurofibromin was seen in the nuclei and/or cytoplasm of all NF1-associated BC specimens, but without any discernable consistent pattern in the intensity or extent of staining. It appears that their aggressive features are related to germline *NF1* mutations in cooperation with somatic mutations in *TP53, KMT2C* and other genes.

Chapter 4: Immunohistochemical expression of neurofibromin in sporadic breast cancers

From the initial discovery cohort of 314 sporadic breast cancers of all subtypes, tumours with both nuclear and cytoplasmic expression of neurofibromin seemed to have better outcomes, especially in the triple negative subset. However, there was no correlation between expression of neurofibromin and survival outcomes in a larger validation cohort of triple negative breast cancers.

Chapter 5: Elucidating therapeutic molecular targets in premenopausal Asian women with recurrent breast cancers

Targeted sequencing was performed on a separate cohort of premenopausal poor prognosis BCs. The most prevalent alterations included *TP53* (65%). *PIK3CA* (32%), *GATA3* (29%), *ERBB2* (27%), *MYC* (25%) and *KMT2C* (21%). The frequency of *NF1* mutations was 2%. Detecting changes in dosage of the *NF1* gene in formalin-fixed paraffin-embedded specimens was not feasible.

Chapter 6: Conclusion and future directions

The final chapter summarises the findings of these studies and highlights future directions that are clinically relevant.

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List of Publications Contributed to During Candidature

1. **Yap Y-S**, Wong J, Chan M, et al. Abstract P3-08-09: Clinical and Pathological Characteristics of Breast Cancer in Women with Neurofibromatosis Type 1. *Cancer Res.* 2012;72 (24 Supplement):P3-08-09-P03-08-09.

2. **Yap YS**, McPherson JR, Ong CK, Rozen SG, Teh BT, Lee AS, Callen DF. The NF1 gene revisited - from bench to bedside. Oncotarget. 2014 Aug 15; 5(15):5873-92.

3. McPherson JR, Ong CK, Ng CC, Rajasegaran V, Heng HL, Yu WS, Tan BK, Madhukumar P, Teo MC, Ngeow J, Thike AA, Rozen SG, Tan PH, Lee AS, Teh BT, **Yap YS**. Whole-exome sequencing of breast cancer, malignant peripheral nerve sheath tumor and neurofibroma from a patient with neurofibromatosis type 1. Cancer Med. 2015 Dec; 4(12):1871-8.

4. Koczkowska M, Chen Y, Callens T, Gomes A, Sharp A, Johnson S, Hsiao MC, Chen Z, Balasubramanian M, Barnett CP, Becker TA, Ben-Shachar S, Bertola DR, Blakeley JO, Burkitt-Wright EMM, Callaway A, Crenshaw M, Cunha KS, Cunningham M, D'Agostino MD, Dahan K, De Luca A, Destrée A, Dhamija R, Eoli M, Evans DGR, Galvin-Parton P, George-Abraham JK, Gripp KW, Guevara-Campos J, Hanchard NA, Hernández-Chico C, Immken L, Janssens S, Jones KJ, Keena BA, Kochhar A, Liebelt J, Martir-Negron A, Mahoney MJ, Maystadt I, McDougall C, McEntagart M, Mendelsohn N, Miller DT, Mortier G, Morton J, Pappas J, Plotkin SR, Pond D, Rosenbaum K, Rubin K, Russell L, Rutledge LS, Saletti V, Schonberg R, Schreiber A, Seidel M, Siqveland E, Stockton DW, Trevisson E, Ullrich NJ, Upadhyaya M, van Minkelen R, Verhelst H, Wallace MR, **Yap YS**, Zackai E, Zonana J, Zurcher V, Claes K, Martin Y, Korf BR, Legius E, Messiaen LM. Genotype-Phenotype Correlation in NF1: Evidence for a More Severe Phenotype Associated with Missense Mutations Affecting NF1 Codons 844-848. Am J Hum Genet. 2018 Jan 4; 102(1):69-87.

5. **Yap YS**, Munusamy P, Lim C, Chan CH, Prawira A, Loke SY, Lim SH, Ong KW, Yong WS, Ng SBH, Tan IBH, Callen DF, Lim JCT, Thike AA, Tan PH, Lee SG. Breast cancer in women with neurofibromatosis type 1 (NF1) – a comprehensive case series with molecular insights into its aggressive phenotype. Breast Cancer Res Treat. 2018 Jun 21. Epub ahead of print.

6. **Yap YS**, Singh AP, Lim JHC, Ahn JH, Jung KH, Kim J, Dent RA, Ng RCH, Kim SB, Chiang DY. Elucidating Therapeutic Molecular Targets in Premenopausal Asian Women with Recurrent Breast Cancers. Accepted for publication in npj Breast Cancer, 2018.

Abbreviations

2ME2: 2-methoxyoestradiol

AC: adenyl cyclase

Akt: protein kinase B, or V-akt murine thymoma viral oncogene homologue 1

ATRA: all-trans retinoic acid

BC: Breast Cancer

BCR-ABL:

BMP2: Bone Morphogenetic Protein 2

BRCA1: breast cancer gene 1

cAMP: cyclic adenosine monophosphate

Cav-1: caveolin-1

CI: confidence interval

CNS: central nervous system

CSRD: cysteine-serine-rich domain

DDR: DNA damage response

EMT: epithelial mesenchymal transition

ER: oestrogen receptor

EVI2A, EVI2B: ecotropic viral integration sites

FAK: focal adhesion kinase

FFPE: formalin-fixed paraffin-embedded

FTS: farnesylthiosalicyic acid

GAP: GTPase-activating protein

GDP: guanosine diphosphate

GIST: gastrointestinal stromal tumour

GPCRs: G-protein coupled receptors

Grb2: growth factor receptor bound 2

GRD: GAP-related domain

GRD2: domain II-related GAP

GTP: guanosine triphosphate

HER2: human epidermal growth factor receptor 2

HR: hormone receptor

HR: hazard ratio

HSF: heat shock factor

ICGC: International Cancer Genome Consortium
JMML: juvenile myelomonocytic leukaemias
LIMK: LIM kinase
LOH: Loss of heterozygosity
MAPK(also known as MEK/ERK): mitogen-activated protein kinase
MEFs: mouse embryonic fibroblasts
MNK: MAPK-interacting kinase
MPNST: malignant peripheral nerve sheath tumour
mTOR: mammalian target of rapamycin
NCCS: National Cancer Centre Singapore
NF1: Neurofibromatosis Type 1
NF2: Neurofibromatosis Type 2
NSCLC: non-small cell lung cancer
OMGP: oligodendrocyte-myelin glycoprotein
OPG: optic pathway gliomas
PAK1: P21-Activated Kinase
PD-1: Programmed cell death protein 1
PDGFRB: Platelet-derived growth factor receptor beta precursor
PI3K: phosphoinositide 3-kinase
PR: progesterone receptor
Rac1: Ras-related C3 botulinum toxin substrate 1
RAF: murine sarcoma viral oncogene homologue
RAS: rat sarcoma viral oncogene homologue
ROCK: Rho-associated kinase
RTKs: receptor tyrosine kinases
SOS: mammalian homolog of the Drosophila son of sevenless
STAT3: signal transducer and activator of transcription 3
TCGA: the Cancer Genome Atlas
TMA: tissue microarray
TOP2A: topoisomerase II alpha
TP53: tumour protein p53
XIAP: X-linked inhibitor of apoptosis

Chapter 1

The NF1 gene revisited – from bench to bedside

Literature review on NF1 and cancer

This chapter is an updated version of our paper, **"**The NF1 gene revisited - from bench to bedside" published in Oncotarget. 2014, with the addition of recent findings.

1.1 Abstract

Neurofibromatosis type 1 (NF1) is a relatively common tumour predisposition syndrome related to germline aberrations of *NF1*, a tumour suppressor gene. The gene product neurofibromin is a negative regulator of the Ras cellular proliferation pathway, and also exerts tumour suppression via other mechanisms.

Recent next-generation sequencing projects have revealed somatic *NF1* aberrations in various sporadic tumours. *NF1* plays a critical role in a wide range of tumours. *NF1* alterations appear to be associated with resistance to therapy and adverse outcomes in several tumour types.

Identification of a patient's germline or somatic *NF1* aberrations can be challenging, as *NF1* is one of the largest human genes, with a myriad of possible mutations. Epigenetic factors may also contribute to inadequate levels of neurofibromin in cancer cells.

Clinical trials of *NF1*-based therapeutic approaches are currently limited. Preclinical studies on neurofibromin-deficient malignancies have mainly been on malignant peripheral nerve sheath tumour cell lines or xenografts derived from NF1 patients. However, the emerging recognition of the role of *NF1* in sporadic cancers may lead to the development of *NF1*-based treatments for other tumour types. Improved understanding of the implications of *NF1* aberrations is critical for the development of novel therapeutic strategies.

1.2 Introduction

Neurofibromatosis type 1, also known as NF1 or von Recklinghausen's disease, is a tumour predisposition syndrome characterized by the development of multiple neurofibromas, café-au-lait spots and Lisch nodules. Initially described by Professor Von Recklinghausen, a German pathologist back in 1882, NF1 is one of the most common genetic disorders worldwide [1,2]. The *NF1* gene is a classic tumour suppressor gene on chromosome 17. Its product neurofibromin is an important negative regulator of Ras cellular proliferation pathways [3-7]. Individuals with NF1 are at increased risk of developing various tumours, including malignant peripheral nerve sheath tumour (MPNST), phaeochromocytoma, leukaemia, glioma, rhabdomyosarcoma and breast cancer [8,9]. Neurofibromatosis type 1 or NF1 is distinct from neurofibromatosis type 2 (NF2), which is less common. NF2 syndrome is related to mutations in *NF2* on chromosome 22, with a different spectrum of tumours, notably schwannomas, meningiomas and ependymomas [10].

More recently, somatic *NF1* aberrations have been increasingly reported in various sporadic tumours, including brain, lung, breast, ovarian tumours and melanomas. Significant challenges remain in the detection of both germline and somatic aberrations. A better understanding of the implications of these aberrations is critical for the improvement of treatment outcomes of tumours with *NF1* aberrations.

1.3 NF1 syndrome

NF1 is a relatively common genetic condition, with an incidence of approximately 1 in 2,000 to 1 in 5,000 individuals worldwide [2]. Although it is an autosomal dominant genetic disorder, approximately half of the cases have no family history, with the condition arising from sporadic mutations of the *NF1* gene. The germline *NF1* mutation rate is ten-fold higher than that observed in other inherited disease genes, with estimates from 1/7,800 to 1/23,000 gametes [2,11].

The condition has 100% penetrance but its degree of expression varies considerably, even within the same family with the identical mutation [12]. NF1 is diagnosed clinically

for most patients, with genetic testing reserved for equivocal cases or in the context of research studies. The National Institutes of Health (NIH) diagnostic criteria stipulate that at least 2 of the criteria in Table 1.1 must be fulfilled to make the clinical diagnosis of NF1 [13].

Loss-of-function mutations in the *NF1* gene can also lead to the development of a wide range of abnormalities in the cardiovascular, musculoskeletal and nervous systems, in addition to the predisposition to benign and malignant tumours. Hypertension, vasculopathy, valvular dysfunction, skeletal anomalies, dysmorphic features, osteoprorosis, cognitive impairment and epilepsy may occur as part of the NF1 syndrome [14].

Table 1.1: National Institutes of Health (NIH) diagnostic criteria for neurofibromatosis type 1 (NF1)

- Six or more café-au-lait macules >5mm in greatest diameter in prepubertal individuals, and >15mm in postpubertal individuals
- Two or more neurofibromas of any type or one plexiform neurofibroma
- Freckling in the axillary or inguinal regions
- Optic glioma
- Two or more iris hamartoma (Lisch nodules)
- Distinctive bony lesion such as sphenoid dysplasia, or thinning of the long bone cortex with or without pseudoarthrosis
- A first-degree relative (parent, sibling or offspring) with NF1 based on the above criteria

The NF1 phenotype is highly variable, ranging from a very mild manifestation of the disease in certain individuals, to a very severe form in some others [12]. In general, there is no definite correlation between a particular alteration and phenotype. Exceptions include deletion of the entire *NF1* gene which is associated with a severe form of the disease [15,16], a recurrently ascertained 3-bp in-frame deletion of exon 17 (c.2970-2972 delAAT) that is associated with the typical pigmentary NF1 features but without cutaneous or surface plexiform neurofibromas [17], and duplication of the *NF1* locus which usually leads to intellectual impairment and epilepsy without the other NF1

features [18,19]. More recently, *NF1* missense mutations affecting arginine at position 1809 were reported to be associated with learning disabilities, pulmonic stenosis, and Noonan-like features, but no external plexiform neurofibromas or symptomatic optic pathway gliomas (OPGs) [20,21]. In addition, missense mutations affecting one of five neighboring *NF1* codons—Leu844, Cys845, Ala846, Leu847, and Gly848 located in the cysteine-serine-rich domain (CSRD). These individuals have a high prevalence of plexiform and symptomatic spinal neurofibromas, symptomatic optic pathway gliomas (OPGs), other malignant neoplasms, and bone abnormalities. These findings demonstrate that missense mutations outside the GAP-related domain (GRD) can be associated with a severe phenotype [22]. There may also be intra- and interfamilial variation in the severity of the phenotype, suggesting that expression of the same genotype may be influenced by epigenetic or environmental factors [12,23]. Females with NF1 often experience an exacerbation of the condition following pregnancy, possibly related to changes in the hormonal milieu [24].

This overview will focus on mainly the oncological aspects of *NF1* aberrations, given the recent discovery of somatic *NF1* aberrations in various cancers in individuals without germline NF1.

1.4 Biology of *NF1* and neurofibromin

Identified and cloned in 1990, the *NF1* gene is located at chromosome 17q11.2 [4,25], and is one of the largest genes in the human genome, with 60 exons spanning over 350kb of genomic DNA [4,26]. Another distinctive feature of the gene is the presence of 3 genes in intron 27b on the antisense strand: OMGP (oligodendrocyte-myelin glycoprotein), a membrane glycoprotein, and EVI2A and EVI2B (ecotropic viral integration sites), which are involved in the development of mouse leukemia [27,28].

NF1 encodes the protein neurofibromin, which has an estimated molecular mass of 327kDa and consists of 2818 amino acids. Neurofibromin is ubiquitously expressed, but most highly in the central nervous system, especially in neurons, astrocytes, oligodendrocytes and Schwann cells [29]. As might be expected for such a large gene, alternate exons, splice variants and alternate start sites have been reported. The

major reported functional isoforms are derived from the insertion of extra exons that preserve the open reading frame and show tissue restricted expression.

The two major isoforms are neurofibromin types I and II. Neurofibromin type I is expressed predominantly in the brain, and has significant Ras regulatory activity. Neurofibromin type II, also known as GRD2 (domain II-related GAP), is the product of the insertion of exon 23a. In contrast to neurofibromin type 1, it has limited GTPase-activating protein (GAP) regulatory function [30,31]. It is expressed mainly in Schwann cells, and is essential for learning and memory in mouse models. In studies on sporadic colon, ovarian and breast cancers as well as gastric cancer cell lines, expression of the type I isoform relative to type II isoform is increased in tumour samples compared to normal tissue [32-35].

Information on other neurofibromin isoforms is limited. Neurofibromin types III and IV, which contain exon 48a and both exons 23a and 48a respectively, are expressed in mainly cardiac and skeletal muscles. They appear to be essential for normal muscle and cardiac development [36,37]. Apart from neurofibromin types I-IV, two other isoforms have been described. An isoform which contains exon 9a is expressed mainly in neurons of the forebrain, and may be involved in memory and learning mechanisms [38,39]. Another isoform has alternative exon 10a-2 inserted, introducing a transmembrane domain. The function of this variant, which is observed in a majority of human tissues, is unclear, but may perform a housekeeping function in intracellular membranes [40].

1.5 Roles of *NF1* and neurofibromin in tumour suppression

NF1 is considered a classical tumour suppressor gene, with both copies of the *NF1* gene reported to be inactivated in benign and malignant tumours in NF1 patients [41-43]. The first hit is inherited or acquired as a germline mutation, and the second hit occurs from a somatic event. Loss of heterozygosity (LOH) due to large somatic rearrangements, deletions and somatic recombination may affect the wild-type *NF1* allele. This can also potentially affect other genes on chromosome 17, which include the tumour suppressor protein *p53* at 17p13.2, human epidermal growth factor

receptor 2 (*HER2*) at 17q21.1, topoisomerase II alpha (*TOP2A*)(17q21.1), signal transducer and activator of transcription 3 (*STAT3*)(17q21.2) and breast cancer gene 1 (*BRCA1*)(17q21.2) [44].

Various *Nf1*$^{+/-}$ mouse models show predisposition to tumour formation, including phaeochromocytomas, leukaemias and malignant peripheral nerve sheath tumours (MPNST), similar to the spectrum of NF1-associated malignancies observed in human counterparts [45-47].

The tumour suppressor function of neurofibromin is largely attributed to a small central region which comprises 360 amino acids encoded by exons 20-27a. This critical region has marked structural and sequence similarity to ras-guanosine-triphosphate(GTP)ase activation proteins (GAPs) and is known as the GAP-related domain (GRD). GAPs inactivate Ras by accelerating the conversion of active Ras-GTP to its inactive guanosine diphosphate (GDP)-bound form. The downregulation of oncogene Ras by neurofibromin prevents the downstream activation of mitogen-activated protein kinase (MAPK) and the PI3K/Akt/mTOR (mammalian target of rapamycin) cell proliferation and differentiation pathways, as demonstrated in Figure 1.1 below [3,5-7,48].

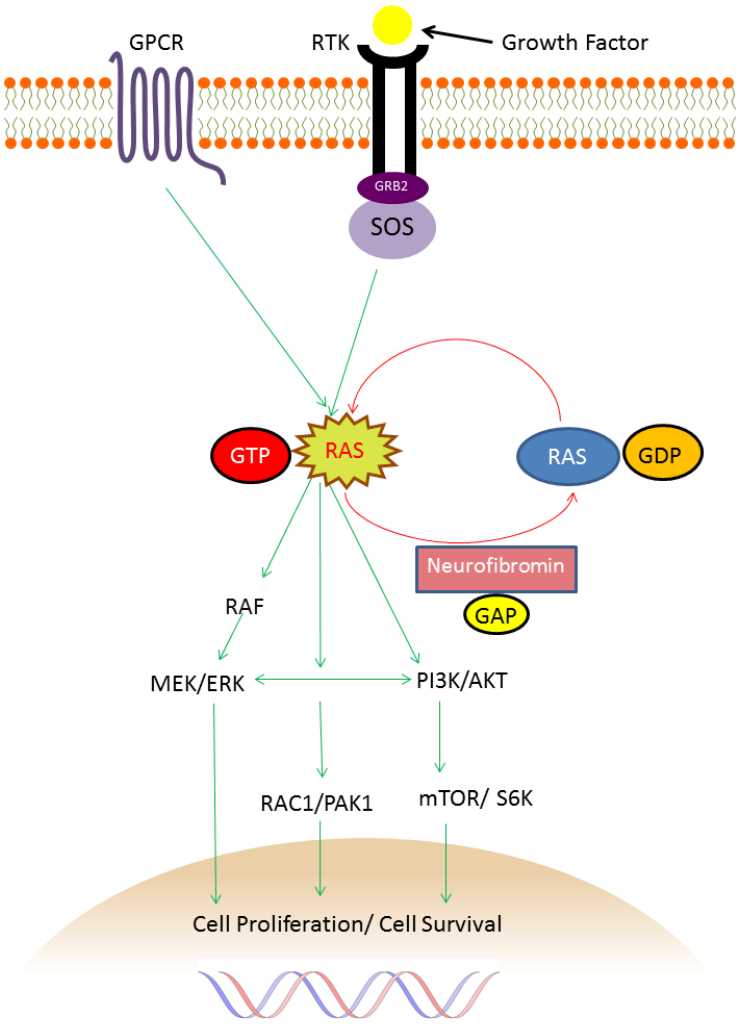

Figure 1.1: The role of *NF1* and neurofibromin in the Ras pathway.

G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs), when activated by ligand, promote guanine nucleotide exchange to form activated Ras-GTP complex. Neurofibromin inactivates Ras by accelerating the conversion of active Ras-GTP to inactive GDP-bound Ras with its Ras-GTPase activity. Consequently, neurofibromin suppresses activation of the downstream effectors of Ras, including PI3K, Akt, mTOR, S6 kinase and RAF, MEK, ERK as well as RAC1 and PAK1.

RTKs=receptor tyrosine kinases. Grb2=growth factor receptor bound 2. SOS=mammalian homolog of the Drosophila son of sevenless. RAS=rat sarcoma viral oncogene homologue. GDP=guanosine diphosphate. GTP=guanosine triphosphate. RAF=murine sarcoma viral oncogene homologue. MEK=MAPK-ERK kinase. PI3K=phosphatidylinositol-3–kinase. AKT=V-akt murine thymoma viral oncogene homologue 1. mTOR=mammalian target of rapamycin. Rac1=Ras-related C3 botulinum toxin substrate 1. PAK1=P21-Activated Kinase.

The Ras-GAP function of neurofibromin may be enhanced by protein kinase C (PKC) phosphorylation of the cystein-serine rich domain (CSRD) of the neurofibromin domain encoded by exons 11-17. The clustering of missense mutations in these regions among NF1 patients indicate the importance of PKC phosphorylation in sustaining normal neurofibromin function [22,49,50].

Neurofibromin has also been demonstrated to bind to caveolin-1 (Cav-1), a membrane protein which regulates signalling molecules such as $p21^{ras}$, protein kinase C and growth factor receptors. Formation of the neurofibromin-Cav-1 complex may lead to inactivation of $p21^{ras}$-GTP and modulation of the $p21^{ras}$/MAPK, PI3K/Akt pathways, controlling cell proliferation and differentiation [51]. The finding that missense mutations resulting in the classic NF1 phenotype frequently occur in exons 28 and 32, which encode the caveolin-binding domains of neurofibromin, supports the role of Cav-1 for neurofibromin function [52,53].

Apart from downregulation of Ras via the homology to GAPs, there are several other postulated mechanisms for the tumour suppressor function of neurofibromin (Table 1.2).

Table 1.2: Mechanisms of Tumour Suppression by Neurofibromin

Mechanisms of Tumour Suppression Reported
• Downregulation of Ras • Positive regulation of adenyl cyclase (AC) • Pro-apoptotic effect (ras-dependent and ras-independent) • Regulation of cell adhesion and motility • Suppression of epithelial mesenchymal transition (EMT) • Suppression of heat shock factor (HSF)

Neurofibromin is a positive regulator of the enzyme adenylyl cyclase (AC), which generates intracellular cyclic AMP (cAMP). cAMP-dependent signaling appears to be important in learning and memory, but also provides a possible mechanism for tumour suppressor function as it regulates Ras activity [54,55]. Increased cAMP leads to activation of Rap1, an anti-mitogenic RAS pathway antagonist, which can result in

inhibition of RAF activation in astrocytes [56,57]. cAMP-mediated regulation of MAPK may have differential effects in different tissues; the mechanisms of cAMP-mediated tumorigenesis in tissues outside the nervous system have not yet been elucidated.

Neurofibromin has also been reported to exert tumour suppressor function via a proapoptotic effect by Ras-dependent and Ras-independent pathways. *Nf1*$^{-/-}$, *Nf1*$^{+/-}$, and *Nf1*$^{+/+}$ mouse embryonic fibroblasts (MEFs) exhibited gene-dosage-related resistance to apoptosis. Neurofibromin-deficient MEFs and human NF1 malignant peripheral nerve sheath tumour (MPNST) cells were more resistant to apoptosis than neurofibromin-expressing MEFs and schwannoma cells. Administration of farnesylthiosalicyic acid (FTS), a Ras inhibitor, increased apoptosis of the neurofibromin-deficient SV40 MEFs and MPNST cells, indicating dependence on the Ras pathway. However, the resistance of neurofibromin-deficient SV40 MEFs and MPNST cells to staurosporine (protein kinase C inhibitor which induces apoptosis), UV irradiation, and vincristine was independent of Ras and cAMP, as demonstrated by the inability of Ras inhibitors or agents that elevate cAMP levels to overcome the resistance. Expression levels of key apoptotic components such as Bcl-2 family proteins, caspases and the X-linked inhibitor of apoptosis (XIAP) were similar in neurofibromin-expressing and neurofibromin-deficient MEFs. The exact mechanism of the Ras-independent proapoptotic effects of neurofibromin remains unclear [58].

The role of neurofibromin in cell motility is important not only for the functioning in neurons, but may also contribute to its tumour suppressor function. Neurofibromin regulates the dynamics and reorganisation of actin filaments via the Rho-ROCK-LIMK2-cofillin pathway, and may be involved in adhesion and signalling at neuronal synapses through its interaction (via its GRD and C-terminal domains) with the transmembrane heparin sulphate proteoglycan syndecan. Lack of neurofibromin triggers the Rho-ROCK-LIMK2-cofilin pathway to alter the organization of actin cytoskeleton, promoting cell motility, invasiveness, and cell-cell adhesion, resulting in the formation of large cell aggregates. This may lead to the formation of multiple neurofibromas in NF1 patients, which consist of aggregates of various cell types, including Schwann cells, fibroblasts, endothelial cells and mast cells on a background of excessive extracellular matrix deposition [59,60].

Another mechanism of tumour suppression by neurofibromin relates to its association with the N-terminal of focal adhesion kinase (FAK), a protein localised at contact sites of cells with extracellular matrix known as focal adhesions. This interaction helps to regulate cellular events including adhesion, proliferation, motility, cellular migration and survival. *Nf1*$^{+/+}$ mouse embryonic fibroblast (MEF) cells exhibited less growth under serum deprivation conditions with reduced adherence on collagen and fibronectin-treated plates, compared to *Nf1*$^{-/-}$ cells [61].

There is also data to suggest that loss of neurofibromin leads to epithelial-mesenchymal transition (EMT). EMT is implicated in tumorigenesis and cancer metastasis. Immunohistochemical analysis and real-time quantitative reverse transcription polymerase chain reaction showed increased expression of EMT-related transcription factors including Snail, Slug, Twist, ZEB1 and ZEB2 in NF1-associated neurofibroma specimens and NF1-derived Schwann cells. Knockdown of *NF1* with siRNA induced the expression of these transcription factors in normal human Schwann cells as well as epithelial-like breast cancer cell lines [62].

More recently, loss of *NF1* has been reported to promote carcinogenesis by activating heat shock factor 1 (HSF1), the master transcriptional regulator of the heat shock response. Knockout of *NF1* in MEFs triggered activation of HSF1, increasing HSF1 levels. This resulted in *Nf1*$^{-/-}$ cells becoming tolerant to proteotoxic stress with proteasome inhibitors and HSP90 inhibitor. This activation of HSF1 relied on dysregulated MAPK signaling. HSF1, in turn, supported MAPK signaling. In *NPcis*$^{+/-}$ mouse models where *Trp53* and *Nf1* genes are disrupted on the same chromosome to develop soft tissue sarcomas resembling human MPNSTs, *Hsf1* knockout impeded NF1-associated carcinogenesis by attenuating oncogenic RAS/MAPK signaling. In cell lines from human malignant peripheral nerve sheath tumors (MPNSTs) driven by *NF1* loss and in surgically excised human MPNSTs, HSF1 was also overexpressed and activated or phosphorylated [63].

1.6 Tumours associated with NF1

Individuals with NF1 are predisposed to developing both benign and malignant tumours throughout life. The risk of malignancy is increased 2.5 to fourfold in NF1

compared to the general population [8,64]. Average life expectancy is reduced by 10-15 years, with cancer being the most common cause of death [2]. In a study based on the Finnish Cancer Registry, the 5-year survival of patients with cancer and NF1, excluding nervous tissue cancers, was also found to be inferior than that of comparable cancer patients without NF1 (54.0% v 67.5%; P = 0.01) [65].

The tumour types individuals with NF1 are at increased risk of developing include both nervous system and non-nervous system tumours. The characteristics of the more common NF1-associated tumours are listed in Table 1.3. Accurate estimation of the relative frequencies of the various tumour types is challenging, as different studies based on hospital data may overestimate the frequency of specific tumours compared to population-based studies. This partly accounts for the wide range of prevalence or incidence figures reported in the literature for various tumours.

Malignant peripheral nerve sheath tumours (MPNSTs), previously referred to as neurofibrosarcomas, are a major cause of morbidity and mortality in NF1. MPNSTs typically arise from malignant transformation of plexiform neurofibromas, and occasionally spinal nerve root or subcutaneous neurofibromas. In NF1 the lifetime risk of developing MPNST is 8-13%, with estimated annual incidence at 0.16%, compared to 0.001% in the general population [9,66,67].

There is a wide range of other NF1-associated tumours including optic pathway gliomas (OPGs), rhabdomyosarcomas, neuroblastomas and juvenile myelomonocytic leukaemias (JMML) in the paediatric setting, as well as gastrointestinal stromal tumour (GIST), phaeochromocytomas and carcinoid tumours in adults. OPGs, like MPNSTs, may occur in both children and adults [9,66,67]. More recently, an increased risk of breast cancer among women with NF1 has also been reported [68,69]. Breast cancer in NF1 patients appears to have an aggressive phenotype in reported case series with limited number of patients [70-72].

NF1 patients are also at an increased risk of developing radiation-induced malignancies. In a study of NF1 patients treated with radiotherapy for optic glioma, the relative risk of second central nervous system (CNS) tumour was 3.04 (95% CI, 1.29 to 7.15) [73]. Hence radiotherapy should be avoided in children with NF1, unless it is

absolutely essential. In mouse model studies, *Nf1*$^{+/-}$mice subjected to irradiation developed in-field tumours associated with NF1 such as phaeochromocytomas, as well as typical second malignant neoplasms such as sarcomas and breast cancers [74-76]. This may be related to upregulated, perturbed cell cycle and DNA repair pathways with *NF1*-haploinsufficiency, as observed in human lymphoblastoid cell lines from NF1-affected and normal individuals, as well as in lymphocytes from wildtype and *Nf1*$^{+/-}$ mice. Activation of DNA damage response (DDR) genes can paradoxically trigger oncogene-induced DNA damage and genomic instability, resulting in carcinogenesis [77,78]. Interestingly, somatic monoallelic loss of *NF1* and *TP53* in the adjacent allele was observed in radiation-induced malignancies arising in both wildtype and *Nf1*$^{+/-}$ mice in one study [74]. *NF1* loss appears to be a critical event in mutagen-induced malignancies beyond the classical NF1-associated tumour types.

Table 1.3: Tumours associated with NF1 syndrome

Tumour Type Associated with NF1	Age category	Frequency	Mechanism(s)	Differences compared to sporadic tumours	References
Malignant peripheral nerve sheath tumour (MPNST)	Adult, Paediatric	Lifetime risk 8-13%	LOH of *NF1*, mutation in *TP53*, *TYK2, SUZ12* copy number alterations, including deletion of *CDKN2A,* loss of *PTEN, EED, SUZ12*	Earlier onset; central rather than peripheral location	2,9,66,67,79-90
Optic pathway glioma (OPG)	Adult (usually young), Paediatric	Incidence 1.5%-7.5%; prevalence 5-25%	LOH of *NF1*, mutation in *TP53*, deletion of *CDKN2A*	Earlier onset; anterior rather than posterior optic pathway	9,66,67
Rhabdomyosarcoma	Paediatric	Prevalence 1.4-6%	unknown	Earlier onset; urinary tract rather than head and neck	9,66,91
Neuroblastoma	Paediatric	Unknown	LOH of *NF1*, amplification of *MYCN*, deletion of 1p36		9,92,93
Juvenile myelomonocytic leukaemia (JMML)	Paediatric	Lifetime risk 200-fold increased	LOH of *NF1*, or compound heterozygous microlesions.		67,94-96
Gastrointestinal Stromal Tumours (GISTs)	Adult	Lifetime risk 6%	LOH of *NF1*, some copy number alterations	Small intestine and multiple rather than gastric origin; lack of response to imatinib with lack of *KIT* and *PDGFRA* mutations	9,66,97,98
Phaeochromocytoma	Adult	Prevalence 1%	LOH of *NF1*	Earlier onset; occasionally bilateral or extradrenal	9,66,67,99-101
Carcinoid	Adult	Prevalence 1%	LOH of *NF1*	Earlier onset; periampullary rather than small intestine	66,67,102
Breast Cancer	Adult	Standardised incidence ratio of 3.5 to 5.2	Unknown	Earlier onset; possibly more aggressive	68-72

1.7 Somatic *NF1* Aberrations in Sporadic Tumours and Effects of *NF1* Deficiency

Recent cancer genome sequencing projects have unraveled the heterogeneity of cancer genomes. Somatic *NF1* aberrations are increasingly reported in various sporadic tumours, including brain, lung, breast, ovarian tumours as well melanomas and leukemias (Figure 1.2). This is particularly relevant with the advent of novel molecular therapies which can potentially be targeted at aberrations in the *NF1* pathway. Improved understanding of the mechanisms of carcinogenesis is critical for the optimisation of these targeted therapies.

Brain Tumours

In glioblastoma multiforme (GBM), *NF1* is one of the most frequently mutated or deleted genes. The prevalence of *NF1* somatic mutations in sporadic GBMs was initially estimated to be approximately 15%, with a subsequent study by The Cancer Genome Atlas (TCGA) network reporting aberrations in at least 23% (47 out of 206) of human GBM samples when both *NF1* inactivating mutations and deletions (including heterozygous deletions) were analysed [103,104]. However, when only mutations and homozygous deletions are considered, the frequency of alterations ranges from 12.1 to 17.6% [104,105].

Data from mouse models support the importance of *NF1* as a glioblastoma suppressor gene. Inactivation of *TP53* and *PTEN* may cooperate with *NF1* loss to induce malignant transformation [106]. Haploinsufficiency for the *NF1* tumour suppressor may have functional consequences, such as increased astrocyte proliferation and augmentation of angiogenesis in $Nf1^{+/-}$ heterozygous mouse models [107,108]. Integrated genomic analysis of the TCGA data identified GBMs with *NF1* and *PTEN* alterations to have a distinct mesenchymal-like expression profile. This mesenchymal subtype was characterised by the expression of mesenchymal markers such as *CHI3L1* (also known as *YKL40*) and *MET*, as well as astrocytic markers (*CD44, MERTK*), reflecting epithelial-to-mesenchymal transition. There was also high expression of genes in the tumour necrosis factor (TNF) and NK-κB pathway, related to the greater necrosis and associated inflammatory response in this subtype [109,110].

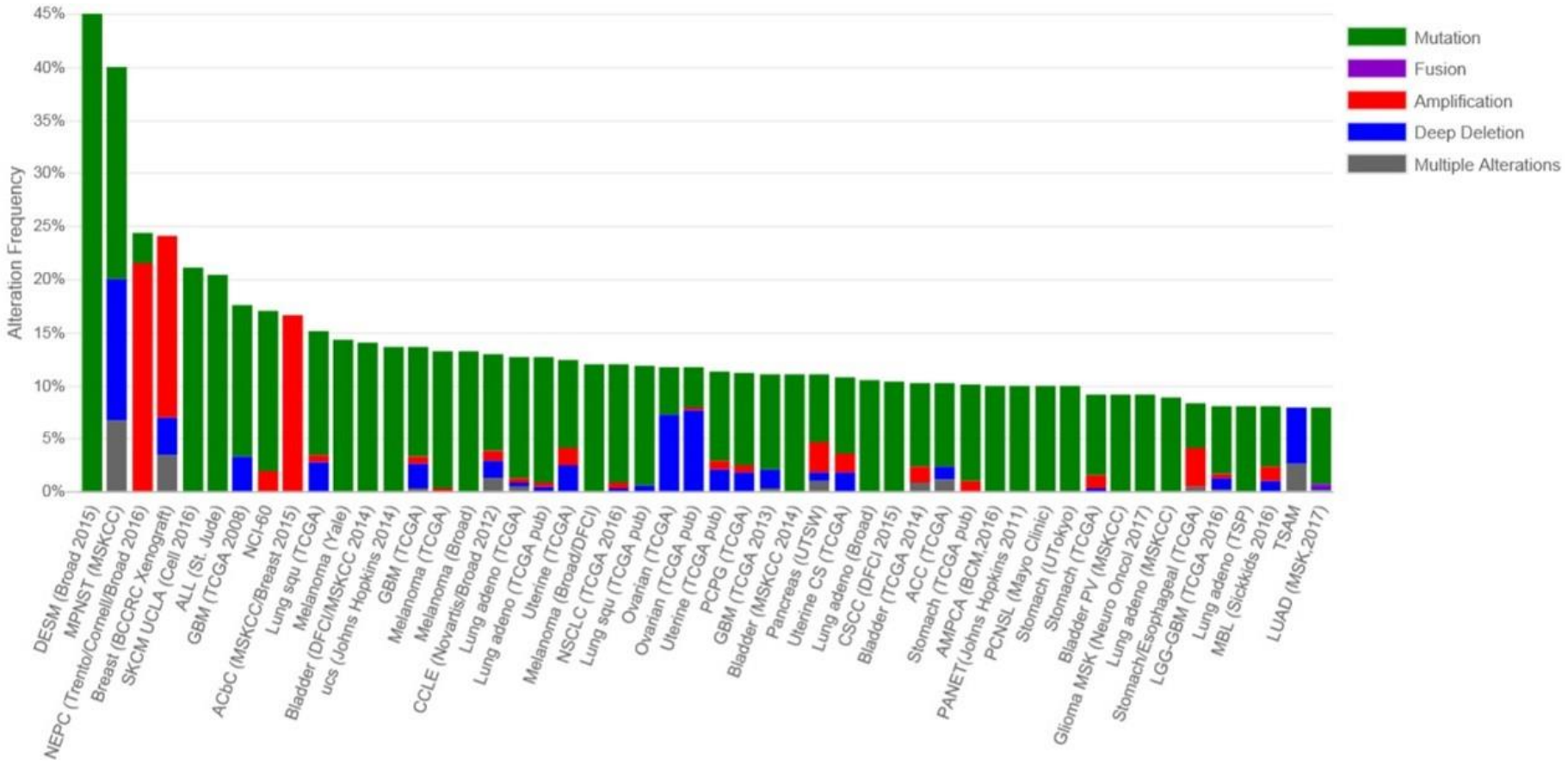

Figure 1.2: Frequency of *NF1* mutation and copy number variation in human neoplasms (Source: The cBio Cancer genomics Portal; http://www.cbioportal.org)

Melanoma

Loss of *NF1* in malignant melanoma cell lines was reported soon after discovery of the *NF1* gene in the early 1990s [111,112], but it was only recently that comprehensive genomic characterization of melanomas was performed. Melanomas may be classified into 3 major classes: 1) sun-shielded melanomas with wild type *BRAF* and *NRAS* which have low mutation load but high number of copy gains, 2) sun-exposed melanomas with *BRAF* or *NRAS* mutations and 3) sun-exposed melanomas with wild-type *BRAF* and *NRAS*, few copy number alterations but high mutation load. The last subtype of melanoma is typically associated with more advanced age, and 30% of melanomas from this class (10/33 samples) carry deleterious *NF1* mutations. *TP53, ARID2* and *PTPRK* are frequently mutated in these melanomas, suggesting that inactivation of tumour suppressors contribute to the pathogenesis of these *BRAF* and *NRAS* independent tumours [113,114]. The overall frequency of *NF1* mutations is estimated at 12-14% of cutaneous melanomas in separate studies (Figure 1.2) [113,115]. *NF1* alterations are especially frequent in desmoplastic melanomas, an uncommon variant of melanoma with sarcomatous histology which usually occur in the chronically sun-exposed skin of older individuals. Up to 54.8% of desmoplastic melanomas harbour *NF1* mutations or copy number aberrations according to one study[116].

Somatic *NF1* mutations have also been reported in melanoma specimens harboring *BRAF* mutations [115,117]. In a mouse model study, *NF1* mutations cooperated with *BRAF* mutations in the pathogenesis of melanomas by preventing oncogene-induced senescence [117]. Loss of neurofibromin expression and *NF1* loss-of-function mutations have been reported in melanomas from patients with *de novo* as well as acquired resistance to BRAF inhibitors [117,118]. A pooled RNA interference screen targeting >16,500 genes in a BRAF inhibitor-sensitive melanoma cell line identified *NF1* as the highest ranking gene whose knockdown abrogated the growth inhibitory effects of PLX4720, a BRAF inhibitor [118]. *NF1*-mutant melanomas are unlikely to respond to standard BRAF-targeted therapies but may benefit from drugs targeting the MEK and PI3K pathway instead. In mice injected with *BRAF/NF1*-mutated melanoma cells, there was resistance to vemurafenib, a BRAF inhibitor. In contrast, there was greater sensitivity to MEK inhibitor PD0325901, PI3K inhibitor GDC-0941

and rapamycin, an mTOR inhibitor. Importantly, rapamycin synergized with PD0325901, resulting in tumour regression in the allografts [117].

Lung Cancer

Whole exome or genome sequencing of primary lung adenocarcinomas identified *NF1* as one of the most frequently mutated genes, with an estimated frequency of 10-13% (figure 1.2) [119,120]. The clinical significance of *NF1* mutations in the larger sequencing studies is not reported, but reduced *NF1* mRNA expression was found to confer both intrinsic and acquired resistance to EGFR inhibitors in a separate study. However, somatic *NF1* mutations were not found in the specimens from these patients with resistant tumours (more details in section on challenges of molecular diagnosis of NF1 and *NF1* somatic aberrations) [121].

Up to 15% of squamous cell lung cancers have alterations in *NF1* (figure 1.2). According to a TCGA study on squamous cell carcinomas, mRNA expression profiling identified 4 distinct subtypes of squamous cell lung cancers – classical, primitive, basal and secretory expression subtypes. The basal expression subtype of squamous cell lung carcinoma characteristically showed alterations in *NF1* [122].

In a study comparing the characteristics of *NF1*-mutant non-small cell lung cancers (NSCLCs) with *KRAS* mutant NSCLCs, *NF1* mutant lung cancers harboured more oncogenic alterations such as *BRAF*, *ERBB2*, *KRAS*, *HRAS*, *NRAS* and *TP53* mutations than *KRAS* mutant cancers, though *NF1* mutations and *KRAS* mutations are not mutually exclusive[123]. In a more recent study, loss-of-function mutations of *RASA1*, a Ras-GTPase activating protein (RasGAP), were reported to be significantly enriched in *NF1*-mutated NSCLCs. Co-mutation of *RASA1*/*NF1* was enriched in adenocarcinomas or squamous cell carcinomas from smokers, and exhibited complete mutual exclusivity with *KRAS* and *EGFR* mutations compared with single mutation of either *RASA1* or *NF1*. Importantly, targeting downstream MAPK signaling with MEK inhibition *in vitro* was significantly more potent in NSCLC cells with *RASA1*/*NF1* co-mutation compared to those with single mutation of either *RASA1* or *NF1*, or with cells that harbor oncogenic *KRAS* or *EGFR* mutations[124].

There is less data on small cell lung cancer, but the frequency of mutations in *NF1* was reported as 2.4% and 6.9% in two separate smaller scale studies [125,126].

Ovarian Carcinoma

The importance of *NF1* in ovarian cancer was first reported by Sangha et al [127]. Initial genome-wide screen of DNA copy number alterations (CNAs) identified apparent *NF1* homozygous deletions in 2 out of 36 primary ovarian serous carcinomas. This led to the discovery that 6 out of 18 ovarian carcinoma-derived cell lines had markedly reduced or lacked expression of NF1 protein, with 5 of the 6 cell lines harbouring *NF1* mutations. Alterations in *NF1*, including splicing mutations and homozygous deletions, were identified in 22% (9/41) of the primary ovarian serous carcinomas studied. There was evidence of Ras pathway activation in these tumours and cell lines with *NF1* defects, in the absence of *KRAS* or *BRAF* mutations. *NF1* appears to cooperate with *TP53* mutations which are present in virtually all ovarian serous carcinomas, in carcinogenesis [127].

In the large scale integrated genomic analyses of 489 high grade serous ovarian carcinomas by the TCGA cooperative group, *NF1* has been recognized as one of the most frequently altered genes, with aberrations in 12% of the cases (8% homozygous deletions, 4% mutations) [128]. These alterations affect signaling in the PI3K/Ras pathway, and may have therapeutic implications as discussed later in this review.

Breast Tumours

Although a computational biology study on gene expression datasets had previously reported associations between the activity levels of regulatory pathways linked with *NF1* to clinical outcome in breast cancer [129], the importance of *NF1* in the pathogenesis of breast cancer was not investigated further until recently. Absence of neurofibromin protein and lack of expression of *NF1* mRNA type 1 isoform have been reported in the highly aggressive human breast cancer MDA-MB-231 cell line which is resistant to endocrine and cytotoxic agents. This was associated with accumulation of phosphorylated MAPK and activated Ras [130]. More recently, this Claudin-low subtype cell line was found to harbour an *NF1* mutation [131]. The Cancer Genome Project led

by the Sanger Institute and The Cancer Genome Atlas (TCGA) projects reported *NF1* mutations in approximately 3% of the breast cancers sequenced. Proportionally more *NF1* mutations were found in luminal or ER+HER2- subtypes, although they were also present in selected HER2-overexpressing and triple negative or basal tumours [132,133]. This may have therapeutic implications, given that knockdown of *NF1* in MCF7 cells conferred resistance to tamoxifen in a genome-wide functional study [134]. More recently, *NF1* frame-shift nonsense (FS/NS) mutations have been validated as poor outcome drivers in hormone receptor-positive breast cancers by Griffith et al[135], while inactivating mutations in *NF1* were associated with inferior breast cancer specific survival in ER negative tumours from the METABRIC study which extensively profiled more than 2,000 breast tumours (hazard ratio 2.7, CI1.3–5.5) [136]. The recent findings of increased frequency of *NF1* mutation in metastatic breast cancer lesions in two separate reports, ranging from 7% to 18% of ER positive metastatic lesions, also supports the role of *NF1* mutations in the metastatic process [137,138].

NF1 has been implicated as a breast cancer driver in a mouse model study. *Chaos3* mice, which are engineered with a point mutation in the minichromosome maintenance 4 (Mcm4) gene, are highly unstable genomically, leading to the development of mammary tumours which resemble human breast cancers [139]. *NF1* was found to be deleted in nearly all the mammary tumours from these mouse models. This led to re-examination of the TCGA data. 27.7% of human breast cancers in the TCGA project were subsequently found to harbour *NF1* aberrations, majority of which were heterozygous deletions. Over 40% of HER2-overexpressing and basal subtypes showed these aberrations. This highlights the importance of investigating genomic loss in addition to somatic mutation of *NF1* [139].

Loss of heterozygosity of *NF1* has been detected in radiation-induced breast cancers from patients without NF1 syndrome. The monoallelic loss of *NF1* is likely to increase the potential for cooperating with other pathways such as *TP53* pathways to promote cellular proliferation and carcinogenesis [74]. Loss of *NF1* gene has also been reported in malignant phyllodes tumour of the breast [140] .

Haematological Malignancies

NF1 was previously implicated as one of the important drivers in certain sporadic haematological malignancies. Myeloid malignanices frequently harbor mutations in the Ras pathway. It is likely that *NRAS/KRAS/NF1* aberrations cooperate with mutations in transcription factors and genes that regulate the epigenome in complex events leading to the development of AML [141]. In earlier studies, *NF1* mutations were reported in up to 7% of acute myeloid leukemia (AML) cases, while 12% of 95 cases studied had copy number alterations in *NF1* with mainly heterozygous deletions. Complete absence of *NF1* expression was reported in 7% of adult AML, and this was associated with increased Ras-bound GTP [142]. In another study on a subset of AML with *CBFB–MYH11* rearrangements, 16% of cases showed deletion of *NF1* [143]. However, two recent large scale studies suggested that *NF1* aberrations are not as frequent in de novo AML, although it may occur as a secondary event in disease progression [144,145]. After taking into account the size of the gene in the test for significantly mutated genes, *NF1* is not one of the significantly mutated genes in AML, with the gene altered in 2.7% of 187 cases [145].

Limited data suggests the frequency of *NF1* alterations in myelodysplastic syndrome (MDS) varies from 0% to 9% [146,147]. Recurrent cryptic alterations or deletions of the *NF1* locus have been detected in 3 out of 35 patients in one of the studies [146]. The frequency of *NF1* mutations in sporadic acute lymphoid leukemia (ALL) was recently reported as 3-8% [148,149]. The prevalence of *NF1* aberrations in certain paediatric haematological malignancies may be higher, with mutation frequency in *NF1* as high as 20% (Fig 1.2).

Colorectal Carcinoma

Data on the nature and the frequency of *NF1* aberrations in colorectal carcinoma vary widely. After the initial report by Li et al that 1 out of 22 sporadic colon adenocarcinomas (4.5%) harboured the amino acid substitution altering Lys-1423 in the *NF1* GRD [150], loss of heterozygosity (LOH) involving the *NF1* gene in 14-57% of colorectal carcinomas was reported in two small studies [151,152]. In addition to *NF1* missense mutations, Ahlquist et al also found duplication of the whole *NF1* gene or parts of it in 4 out of 24 specimens (17%) [153]. Nine out of ten *NF1* mutations detected in this study occurred in introns likely involved in exon splicing. Notably, 8 of these 10

carcinomas showed microsatellite instability[153]. In contrast, *NF1* was found to be altered in only 3.8-5.6% of colorectal carcinomas in TCGA sequencing studies [154,155].

Other Sporadic Tumours

As displayed in Figure 2; there are several other tumours in which *NF1* aberrations have been reported.

MPNSTs can also be sporadic, that is, occur in individuals without NF1 disorder, or occur after radiotherapy as a treatment-related complication. Somatic alterations of *NF1* occur frequently in 72% of non-NF1-associated MPNSTs, and potentially cooperate with *CDKN2A* somatic alterations (81% frequency among all MPNSTs) and *PRC2* mutations (70% (19/27) of NF1-associated, 92% (12/13) of sporadic and 90% (8/9) of radiotherapy-associated MPNSTs) in the pathogenesis of MPNSTs[89].

Aberrations of *NF1* have been reported in other sporadic soft tissue sarcomas as well. Up to 10.5% of myxofibrosarcomas and 8% of pleomorphic liposarcomas harbor *NF1* mutations [156,157]. In a study on embryonal rhabdomyosarcoma, loss of *NF1* occurred in 35%(9/26) of tumours (heterozygous or homozygous deletion of *NF1* or heterozygous chromosomal loss), and were mutually exclusive with *Ras* mutations, suggesting *NF1* loss as an alternative and potentially common driver of Ras activation in this major subtype of soft tissue sarcoma in young children [157].

A few studies reported somatic *NF1* aberrations or inactivation in 26-41% of sporadic phaeochromocytomas from individuals without NF1. In keeping with the observation that NF1 individuals are at increased risk of developing phaeochromocytomas, these findings suggest that loss of *NF1* function is a crucial event in the pathogenesis of both sporadic and NF1-associated phaeochromocytomas [101,158,159] .

Inactivating *NF1* mutations occur in approximately 11% of urothelial bladder carcinomas sequenced in TCGA study [160]. Alterations in *NF1* appear to be most common in the “neuronal” subtype based on mRNA expression, with a frequency of 10% compared to 1-6% in other molecular subtypes. The neuronal showed relatively

high expression of neuronal differentiation and development genes, and was associated with the poorest survival among the different molecular subtypes[161].

1.8 Challenges of molecular diagnosis of NF1 and detection of *NF1* somatic aberrations

The diagnosis of NF1 syndrome is usually established clinically in individuals with constitutional features of the syndrome. Germline *NF1* testing is reserved mainly for equivocal cases, for prenatal diagnosis and in the research setting. Detection of *NF1* mutations or deletions can be highly challenging due to several factors. *NF1* is one of the largest genes, with 60 exons spanning over 350kb of DNA. The gene also has one of the highest mutation rates, with up to half of the mutations being novel mutations. In addition to the myriad of possible lesions with more than 2,800 different germline mutations reported so far[22] and the lack of mutation hotspots, the presence of several pseudogenes can further complicate the molecular diagnosis [11,162-166]. A multi-step protocol involving analysis of genomic DNA and mRNA with RT-PCR, direct sequencing, multiplex ligation-dependent probe amplification (MLPA), and previously using also microsatellite marker analysis and FISH, was required to identify up to 95% of pathogenic mutations in individuals fulfilling the clinical NIH diagnostic criteria [167-169]. Analysis of RNA is essential as splicing mutations may be present in more than 20% of individuals with NF1 syndrome [163,168,169], and may be located deep in introns which may be missed when only exons are studied.

Given the potential difficulties of detecting the pathogenic mutation in individuals with clinical features of NF1, the identification of somatic *NF1* aberrations in sporadic tumours can also pose a significant challenge. While it is possible that the frequency of somatic *NF1* alterations in various tumours is higher than what is currently recognized, the functional significance of the extensive range of missense mutations remains unclear.

Although next generation sequencing (NGS) may be less laborious than direct sequencing, there are also limitations with NGS techniques. Decreased specificity of the capture probes may lead to the capture and enrichment of off-target sequences,

including those from pseudogenes and closely related genes [162]. Exome sequencing alone may not detect splicing mutations or gene rearrangements. Whole genome sequencing combined with transcriptome analysis may be superior, but there are limitations to its applicability in the clinical setting currently due to the general requirement for fresh frozen tissue, complexity of data analysis and cost.

1.9 Downregulation of *NF1* and neurofibromin via other mechanisms

Epigenetic factors, such as gene silencing by microRNAs and DNA methylation, may also influence the expression of *NF1* and neurofibromin, as described below.

microRNAs are endogenous, small noncoding RNAs which can influence their target gene expression post-transcription. Downregulation of *NF1* by microRNA-193b, which is overexpressed in sporadic head and neck squamous cell carcinomas (HNSCC), led to activation of ERK and resulted in tumour progression. Survival outcomes in HNSCC patients whose tumours expressed high levels of miR-193b were inferior compared to patients with low miR-193b expression. Knockdown of miR-193b in HNSCC cells increased *NF1* transcript and protein expression levels, decreased ERK phosphorylation with reduction in cell viability, migration, invasion and tumour formation [170].

There is limited data on methylation changes, but methylation of *NF1* has been found to be the cause of a somatic second-hit inactivation in pilocytic astrocytoma from a patient with NF1 [171].

Excessive proteasomal degradation of neurofibromin can also result in deficiency of this critical tumour suppressor protein [172]. The ubiquitin ligase complex which controls both the regulated destruction and pathogenic destabilisation of neurofibromin was recently identified in glioblastomas as a Cullin 3(Cul3)/kelch repeat and BTB domain-containing 7 complex. Inhibition of Cul3 with Cul3-specific shRNAs suppressed Ras/ERK signaling; agents aimed at blocking neurofibromin destruction may be a potential therapeutic strategy for further development [173].

Given that the expression of *NF1* may be influenced by epigenetic factors, microRNAs [174] and proteasomal degradation [172,173], a proteomics-based approach may help to detect deficiency of neurofibromin. The utility of immunohistochemical staining of neurofibromin has not been fully explored. Complete absence of neurofibromin staining on immunohistochemistry was found in 15-18% of melanomas [117]. However, quantitation of protein expression correlating with treatment outcomes has not been well studied. This is also complicated by the fact that current antibodies available may not be able to distinguish between the normal and mutant neurofibromin protein. Functional studies of "mutant neurofibromin" will be challenging with the huge protein size and myriad abnormalities possible.

The challenges of elucidating the mechanisms of *NF1* deficiency are demonstrated in the recent study on reduced *NF1* expression as a driver of resistance to EGFR inhibitor in lung cancer. *NF1* mRNA expression was reduced in EGFR TKI-resistant lung cancer specimens, but somatic mutations and methylation changes involving *NF1* were not detected. To account for the downregulation of *NF1* mRNA, immunohistochemistry using multiple antibodies was performed, but none of them demonstrated adequate specificity to detect neurofibromin in human lung tissue [121].

1.10 Therapeutic Strategies for NF1 and NF1-associated/deficient malignancies

Management Options for NF1 syndrome and neurofibromas

The management of individuals with NF1 consists mainly of surgical resection of neurofibromas when they cause discomfort or impingement of neighbouring structures such as nerves or spinal cord. There is an unmet need for novel molecular therapies to treat the systemic manifestations in NF1. Although several early phase trials on NF1-associated plexiform neurofibromas have been listed on the clinicaltrials.gov website, many have been terminated or suspended, with very few studies having published their results.

Early clinical trials using thalidomide, 13-cisretinoic acid (CRA) or interferon α-2a to target angiogenesis and differentiation in NF1 patients with plexiform neurofibromas induced at best a minor response in a minority of patients [175,176]. Early phase trials using pirfenidone, an antifibrotic agent drug which targets the stromal contributions, showed similar limited activity in plexiform neurofibromas in adults and children [177,178].

Since Ras is overactivated with dysfunction of *NF1*, subsequent NF1 trials focused on inhibition of Ras. Farnesylation and geranylgeranylation of Ras proteins is essential for translocation to the cell membrane with subsequent activation of the Ras pathway. The activity of tipifarnib, a farnesyl transferase inhibitor, was reported in a phase 1 trial on children with solid tumours or NF1 and plexiform neurofibromas. Stable disease was the best response; no significant regressions were observed [179]. More recently, in a phase 2 placebo-controlled study on children and young adults with NF1 and progressive plexiform neurofibromas, tipifarnib did not prolong the time to progression compared to placebo [180]. Similarly, results from a phase 2 study using sirolimus (rapamycin), an mTOR inhibitor, in NF1 patients with plexiform neurofibroma, did not report any regression of the lesions [181]. Clinical trials using everolimus, a newer generation mTOR inhibitor and other therapies have also been conducted, but with no reports of the trial findings to date. The MEK inhibitor PD0325901 was effective in shrinking plexiform neurofibromas in more than 80% of genetically engineered mice, but data on clinical activity in human subjects is awaited [182]. A number of preclinical studies also suggest the efficacy of MEK inhibitors in NF1-associated MPNSTs or plexiform neurofibromas may be enhanced in combination with photothermal therapy[183], all-trans retinoic acid (ATRA)[184], and inhibition of BMP2 (Bone Morphogenetic Protein 2)[185] or MNK (MAPK-interacting kinase)[186].

Pegylated interferon-α-2b, which has antiproliferative, antiangiogenic and immunomodulatory properties, induced minor response in 29% of young patients with plexiform neurofibromas in a phase I trial [187]. Tumour stabilization or prevention of new lesions may be a more realistic endpoint as dramatic regression of established “benign” tumours is less likely. Although neurofibromas may show LOH in a subset of Schwann cells, the mode of pathogenesis is different from that of malignant tumours [188]. However, imatinib mesylate, an oral kinase inhibitor targeting c-kit and PDGFRβ, was

recently reported to decrease plexiform neurofibromas by 20% or more in 6 out of 36 NF1 patients in a phase 2 trial. This effect may partially be related to targeting cellular phosphor-signalling cascades[189-191]. In contrast, sorafenib which targets c-kit and PDGFRβ as well as RAF, VEGFR2, was poorly tolerated and did not show any tumour response in a phase 1 trial on children with NF1 and plexiform neurofibromas [192]. Based on the preclinical activity of nilotinib (tyrosine kinase inhibitor with activity against BCR-ABL and c-kit among other tyrosine kinases) in NF1-associated MPNST and plexiform neurofibromas[193], a phase 1 trial on adults with NF1-associated plexiform neurofibromas was initiated, with results awaited. The clinical efficacy of these compounds in treating neurofibromas remains to be tested in larger clinical trials.

A novel approach to the treatment of NF1 may potentially involve STX3451 (2-(3-Bromo-4,5-dimethoxybenzyl)-7-methoxy-6-sulfamoyloxy-1,2,3,4-tetrahydroisoquinoline), a small molecule hormone-like agent resembling the natural anticancer metabolite, 2-methoxyoestradiol (2ME2). It may provide a novel approach for lowering the tumour burden in NF1 and other endocrine sensitive cancers with limited treatment options[194]. The number and size of tumours in NF1 patients are known to increase in response to the rise in steroid hormones during puberty and pregnancy, with regression after delivery [24]. This hormonal dependency can potentially be exploited in the treatment of NF1. STX3451 was reported to induce apoptosis in human MPNST cell lines, with inhibition of PI3K-mTOR signaling pathways, as well as disruption of actin- and microtubule-based cytoskeletal structures in MPNST and plexiform neurofibroma cell lines[194].

Potential therapeutic strategies for NF1-deficient malignancies

Data on the efficacy of molecular therapies in NF1-deficient malignancies is currently limited to results from preclinical studies (Figure 1.3). Much of this research has been conducted on models of MPNST derived from NF1 patients. This is set to change with emerging clinical trials where the molecular therapy is matched to the genomic profile of each individual's tumour. A one-size-fits-all approach may not always deliver an optimal outcome. For instance, although imatinib is standard-of-care for most patients with sporadic GIST, *KIT/PDGFRA* mutations are uncommon in GISTs arising in NF1 individuals, so response to imatinib is poor in these patients[98]. With the increasing

personalization of treatment in cancer, there is now an ongoing phase 2 trial using selumetinib in *NF1*-mutated (germline or somatic) GIST (ClinicalTrials.gov Identifier: NCT03109301).

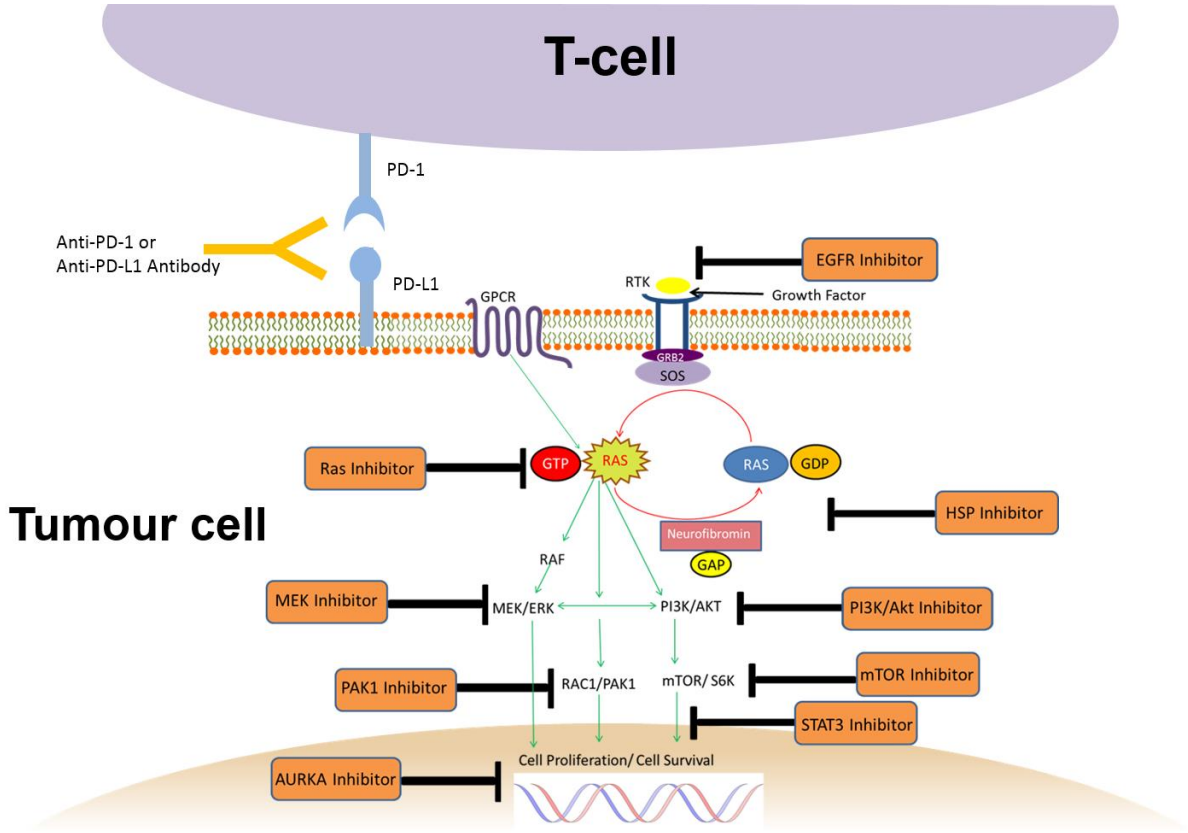

Figure 1.3: Potential therapeutic strategies for NF1-deficient malignancies

The molecular therapies above have been tested in the preclinical setting, largely for MPNSTs. There is also data on some of the inhibitors for neurofibromin-deficient breast cancer, glioblastoma, AML, soft tissue sarcoma, lung cancer and melanoma. Combination therapy targeting more than one checkpoint may be required for optimal inhibition.

There is preclinical data to support the activity of MEK inhibitors, Ras inhibitor farnesylthiosalicylic acid, sirolimus, everolimus and PI3K/Akt/mTOR inhibitors, in MPNST cell lines or xenografts derived from NF1 patients [182,195-199] (Figure 3). The addition of erlotinib, an epithelial growth factor receptor (EGFR) inhibitor to everolimus, inhibited growth and induced apoptosis further in 4 NF1-derived and 1 sporadic MPNST cell lines as well as the STS26T sporadic MPNST xenograft [197]. EGFR expression is present in most MPNST cell lines, and the EGFR signaling pathways

were found to be associated with tumorigenesis in the Nf1:p53 mouse tumor model [197,200].

Signal transducer and activator of transcription-3 (STAT3) is a potential target for treating NF1-associated or NF1-deficient cancers, as STAT3 is activated downstream in the PI3K/mTOR pathway. The natural product cucurbitacin-I, a potent STAT3 inhibitor, was found to inhibit the growth of NF1-deficient MPNST cells in vitro and in vivo in xenografts [201].

Since heat shock factor is activated with loss of NF1, it is not surprising that the addition of HSP90 inhibitor IPI-504, to rapamycin, led to synergistic activity with damage of endoplasmic reticulum and mitochondria in NF1-deficient MPNST mouse models [202].

More recently, integrative transcriptome analyses have identified Aurorakinase A(*AURKA*) as a potential therapeutic target. *AURKA* was overexpressed and amplified in NF1-related MPNST, but not neurofibromas. MLN8237, an AURKA selective inhibitor, was effective in stabilizing tumour volume and prolonged survival of mice with MPNST xenografts [203].

Inhibitors of PAK1, a downstream effector in the Ras pathway, have also been reported to suppress the growth of *NF1*-deficient MPNST cells as well as neurofibromin-deficient human breast cancer (MDA-MB-231) xenografts in mice. There is evidence that many tumours, including breast cancers, are addicted to abnormal activation of PAK1, a Ser/Thr kinase which in turn stimulates cyclin D1, for their growth [204,205].

In sporadic tumours harbouring *NF1* aberrations, MEK inhibitors have been found to be effective in treating neurofibromin-deficient sporadic glioblastoma cell lines, *NF1*-deficient AMLs and *NF1*-deleted soft tissue sarcomas in mouse models [206-208]. Following the discovery that *NF1* deficiency confers intrinsic and acquired resistance to EGFR inhibitor in lung cancer, treatment of neurofibromin-deficient lung cancers *in vitro* and in xenografts with MEK inhibitory drugs (AZD-6244, CI-1040 and PD0325901) restored sensitivity to erlotinib when given in combination [121]. An ongoing trial is

investigating the efficacy of trametinib, an inhibitor of MEK1 and MEK2 in NF1-mutant non-small cell lung cancer (ClinicalTrials.gov Identifier: NCT03232892), while another trial involves the use of selumetinib in young adults with recurrent or refractory low grade gliomas with activation of the MEK pathway (ClinicalTrials.gov Identifier: NCT01089101).

Combination therapies targeting more than one checkpoint in the cell proliferation pathway, such as blocking both the PI3K/mTOR and MEK pathways in the allografts of *NF1/BRAF*-mutated melanomas and dual EGFR, MEK inhibition concurrently in TKI-resistant NF1-deficient lung adenocarcinomas, may be superior to monotherapy [117,121]. Inhibiting a single checkpoint may lead to activation of compensatory negative feedback pathways[209], though cumulative toxicities from blockade of multiple targets can be challenging in clinical practice.

Future strategies may include inhibition of excessive destruction of neurofibromin and other epigenetic therapies. In *Nf2*-mutant Schwann cells, inhibition of SIRT2, a class III histone deacetylase, triggered necrosis [210]. The role of HDAC inhibitors, which may decrease Akt phosphorylation, has not been fully explored for *Nf1*-mutant cells. Inhibition of LIM kinase in the Rho-ROCK-LIMK-cofilin pathway regulated by neurofibromin is another potential strategy. In *Nf1*$^{-/-}$ MEFs, novel LIMK inhibitors blocked the phosphorylation of cofilin, resulting in actin severance and inhibition of cell migration and growth [211]. The utility of these drugs in NF1-deficient tumours may be worth investigating, especially in combination with Ras or AURKA inhibitors, which may have synergistic effects [211,212].

With the recent breakthroughs in immunotherapy for the treatment of several tumour types, immune checkpoint blockade may be promising in the treatment of *NF1*-mutant or deficient tumours, which may also be associated with higher mutation burden. An impressive response rate of 70% was reported recently in 60 patients with desmoplastic melanomas treated with antibodies blocking PD-1 or PD-L1 (PD-1 ligand), with 32% complete response rate. Whole-exome sequencing of 17 cases in this cohort revealed frequent *NF1* mutations (14/17), with high mutational load[213] . While this observation is encouraging, it is unclear if the efficacy is directly related to the *NF1* mutation, or the association with sun damage which may induce more

immunogenic mutations, or a superior adaptive immune response intrinsically with this subtype of melanoma. Improved understanding of the biology of *NF1* and neurofibromin in normal cells and cancer is critical for the development of novel treatment strategies.

1.11 Conclusions

NF1 and neurofibromin play critical roles in tumour suppression. The frequency of somatic *NF1* aberrations in sporadic tumours is increasingly recognized. These alterations are associated with distinct subtypes in certain cancers, and may be associated with poorer treatment outcomes. Significant challenges remain in unravelling the complexity of the large *NF1* gene and its product neurofibromin. Improved molecular diagnosis techniques are essential for detecting these aberrations. There is also an unmet need to develop novel systemic therapies for treating *NF1*-deficient tumours.

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Chapter 2
Whole exome sequencing of multiple tumours from an NF1 patient

2.1 Abstract

Neurofibromatosis type 1 (NF1) is a genetic disorder characterized by the development of multiple neurofibromas, cafe-au-lait spots and Lisch nodules. Individuals with NF1 are at increased risk of developing various tumors, such as malignant peripheral nerve sheath tumour (MPNST), phaeochromocytoma, leukaemia, glioma, rhabdomyosarcoma and breast cancer. Here, we describe the exome sequencing of breast cancer, MPNST and neurofibroma from a patient with NF1. We identified a germline mutation in *NF1* gene which resulted in conversion of leucine to proline at amino acid position 847. In addition, we showed independent somatic *NF1* mutations in all the 3 tumors (frameshift insertion in breast cancer (p.A985fs), missense mutation in MPNST (p.G23R**)**, and inframe deletion in dermal neurofibroma (p.L1876del-Inf)), indicating that a second hit in *NF1* gene resulting in the loss of function could be important for tumor formation. Each tumor had a distinct genomic profile with mutually exclusive mutations in different genes. Copy number analysis revealed multiple copy number alterations in the breast cancer and the MPNST, but not the benign neurofibroma. Germline loss of chromosome 6q22.33, which harbors 2 potential tumor suppressor genes, *PTPRK* and *LAMA2*, was also identified; this may increase tumour predisposition further. In the background of NF1 syndrome, although second-hit *NF1* mutation is critical in tumorigenesis, different additional mutations are required to drive the formation of different tumors.

2.2 Introduction

NF1 is a relatively common genetic disorder characterized by the development of multiple neurofibromas, cafe-au-lait spots and Lisch nodules, with estimated incidence of 1 in 2,000 to 1 in 5,000 individuals worldwide [1]. The *NF1* gene on chromosome 17q11.2 is a classic tumour suppressor gene. Its product neurofibromin is an important negative regulator of the Ras cellular proliferation pathway [2,3]. Individuals with NF1 are at increased risk of developing various tumours, including MPNST, phaeochromocytoma, leukaemia, glioma and rhabdomyosarcoma [4]. More recently, an increased risk of breast cancer has also been reported [5,6].

The mechanism of pathogenesis of NF1-associated breast cancer is unknown; limited data suggest an aggressive biology of breast cancer in NF1 patients [7-9], with a higher proportion of estrogen receptor negative and HER2-posiitve tumours [9,10]. Previously, studies on genetic aberrations in MPNST focused on only a limited set of genes, reporting mutations in *TP53* and second hit *NF1*, multiple copy number alterations, and deletion of *CDKN2A* [4,11-13]. Recently published studies now report frequent somatic aberrations in *EED* and *SUZ12* as well, both of which are chromatin-modifying genes [14-16].

In this study, we sequenced the exomes of breast cancer, MPNST, dermal neurofibroma and matched whole-blood from a single NF1 patient. The objectives were to unravel the genomic complexity of different neoplastic manifestations of NF1 and to identify somatic mutations that potentially drive these tumours.

2.3 Materials and Methods

Patient

The subject of this study fulfilled NIH Consensus Development Conference diagnostic criteria for NF1; family history was also positive for NF1. At the age of 39 years, she was diagnosed with right breast cancer. Histopathological examination revealed a 40mm grade 3 invasive ductal carcinoma with metastasis to 1 of 17 lymph nodes. Estrogen- and progesterone- receptor status were positive. HER2 was 2+ in 30% of the cells by immunohistochemistry, and borderline positive on fluorescent in-situ

hybridization (FISH) testing with a ratio of HER2 to chromosome 17 signals from 60 nuclei scored as 2.2, and an average of 4.7 HER2 signals per nucleus. Adjuvant chemotherapy as well as trastuzumab and tamoxifen were administered postoperatively, in addition to radiotherapy.

Three years later, she presented with a rapidly growing soft tissue mass beneath the right buttock. Excision of this mass and a separate dermal neurofibroma on the right buttock was performed; the pathological diagnosis for the mass was malignant peripheral nerve sheath tumour arising from a plexiform neurofibroma. Sections showed a hypercellular spindle cell tumour with large areas of necrosis and hemorrhage and high mitotic activity.

Blood and fresh frozen tumour specimens (breast cancer, MPNST, dermal neurofibroma) were obtained from this patient who was referred to the candidate YSY for the study. The design of the study was conceived by the candidate YSY who secured grant funding, with approval from the local Institutional Review Board. All the tumour specimens contained at least 70% tumour by routine histologic review with haematoxylin and eosin staining at Singapore General Hospital Pathology Laboratory.

Exome capture and high throughput sequencing

DNA extraction, library preparation and sequencing of DNA were performed by collaborators at the Laboratory of Cancer Epigenome, Division of Medical Sciences, National Cancer Centre Singapore, with bioinformatic analyses by collaborators from the Division of Neuroscience and Behavioral Disorders, Duke-National University of Singapore Graduate Medical School. Three micrograms of DNA per sample were sheared using a Covaris S1 Ultrasonicator (Covaris, MA). Adaptor-ligated libraries were constructed using Paired-End DNA kits (Illumina, CA). Exome capture was performed using SureSelect Human All Exon Kit v3 (Agilent Technology, CA). Each sample was sequenced on two lanes of an Illumina GA-IIx sequencer using 76-bp paired-end reads. The image analysis and base calling were performed using the Illumina pipeline (v1.6) with default settings.

Sequence mapping and coverage computation

Alignment of the sequenced reads was to human reference genome hg19, using the Burrows-Wheeler Aligner software. PCR duplicates were removed using SAMTools. Variants were called using a pipeline based on the Genome Analysis Toolkit (GATK) software. Base quality scores were recalibrated and the sequences near microindels were realigned. Consensus calling for SNVs and microindels was done with the GATK Unified Genotyper. Only well-mapped reads and reads with fewer than 4 mismatches in a 40 base-pair window were considered.

The putative SNVs and microindels were annotated against dbSNP 135 and 1000 Genomes to remove common polymorphisms, excluding cancer-associated positions (based on presence in the COSMIC database). Using transcripts from the CCDS, RefSeq, Ensembl and UCSC databases, we identified non-synonymous mutations and classified them as tumour-somatic if the matched normal sample had sufficient coverage to show that the variant was not present in the germline. Putative mutations were validated by Sanger sequencing.

Copy number variation analysis

Analysis of copy number and regions of loss-of-heterozygosity was performed on the exome sequencing data using the ASCAT algorithm 2.0 (Allele-Specific Copy number Analysis of Tumors) (http://heim.ifi.uio.no/bioinf/Projects/ASCAT/)[17]. For higher resolution, the blood, dermal neurofibroma and primary breast tumour samples were assayed using the Affymetrix CytoScan HD platform. There was insufficient DNA from the frozen specimen of MPNST for the Cytoscan copy number analysis. Analysis of the array data was performed with the Chromosome Analysis Suite software (version 2.0.1) from Affymetrix.

2.4 Results

Whole exome sequencing of breast cancer, MPNST and dermal neurofibroma in NF1 patient identified independent *NF1* mutations.

Our target enrichment and sequencing achieved a mean coverage of 75, with an average of 83% of bases covered by at least 20 reads in each sample (Table 2.1).

Table 2.1: Summary of exome sequencing of the four samples.

Sample Type	Bases in Target Region	Reads Mapped to Target Region*	Ave. Depth Per Targeted Base	Targeted Bases with Depth at Least 1X (%)	Targeted Bases with Depth at Least 20X (%)	Candidate somatic mutations
Normal Blood	51,756,093	81,654,655	90	95.7	85	NA
Breast Cancer	51,756,093	69,567,784	76	95.7	84	15
MPNST	51,756,093	71,616,111	77	95.5	83	6
Neurofibroma	51,756,093	53,446,781	57	95.2	79	1
Average	51,756,093	69,071,333	75	96	83	7

To identify the possible *NF1* germline mutations in this patient, we inspected all variants detected in the patient's blood DNA, and identified a heterozygous missense mutation of thymidine to cytosine (g.chr17:29,556,173). This mutation resulted in a conversion of leucine to proline at amino acid position 847, which is also present in all the tumour samples confirmed by Sanger sequencing, indicating germline mutation (Figure 2.1).

All reported mutations were validated by Sanger sequencing in the 4 samples (blood, breast cancer, MPNST and dermal neurofibroma) (Figure 2.2, Table 2.2), except the insertion of cytosine in the *NF1* gene (g.chr17:29,553,477) in the breast cancer, most probably due to repetitive sequences in that region leading to slippages in Sanger sequencing. However, close inspection using the Integrated Genome Viewer software suggests a true insertion event (Figure 2.3). The somatic mutations identified in each sample were unique to the tumor, suggesting that each different type of tumor arose independently in the patient.

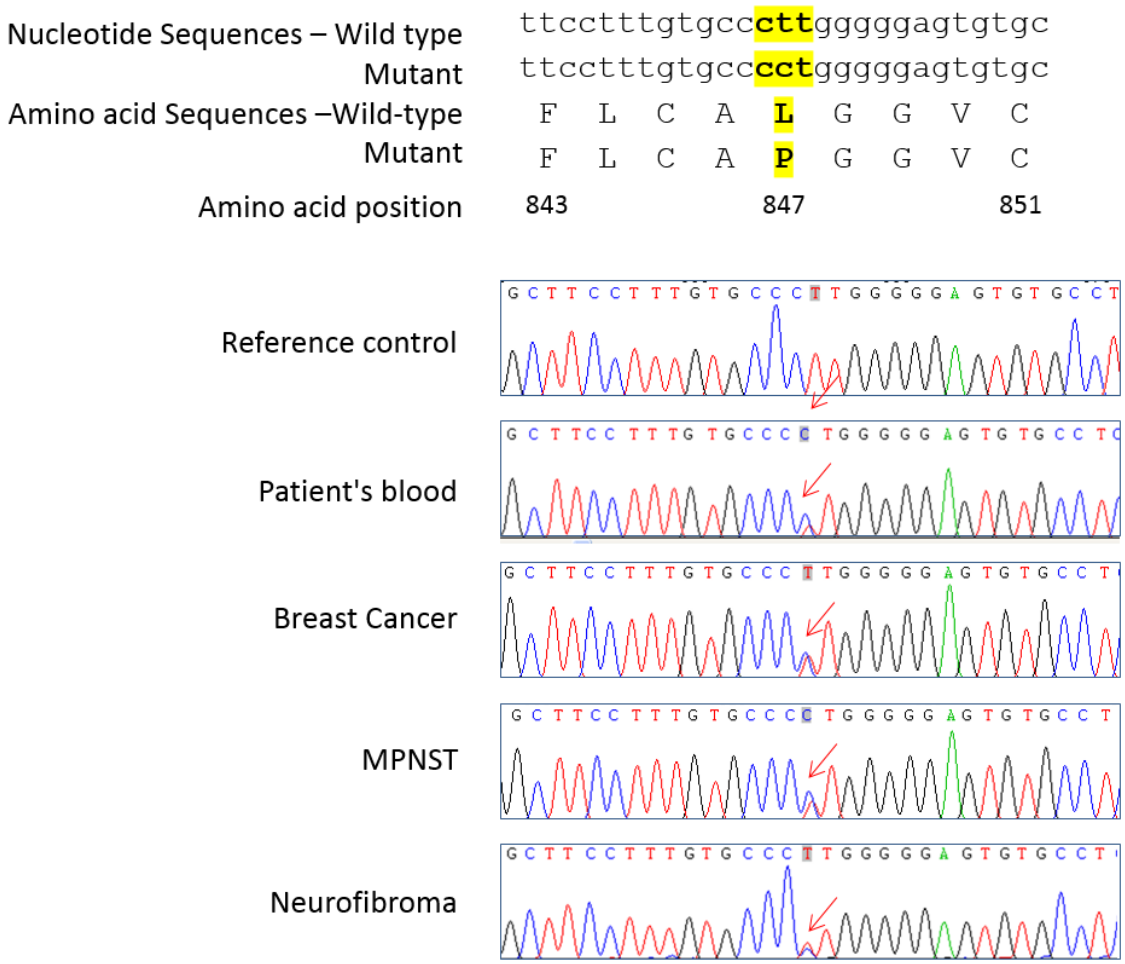

Figure 2.1: Sanger sequencing confirms germline *NF1* variant present in all samples.

The top panel shows the wild type and mutant nucleotide and the altered amino acid sequence at amino acid position 847. The bottom 5 panels are the electropherograms from Sanger sequencing of the same region from a reference control (commercial genomic human DNA), patient's blood, breast cancer, MPNST and dermal neurofibroma. Note that the germline mutation was detected in all samples except the reference control.

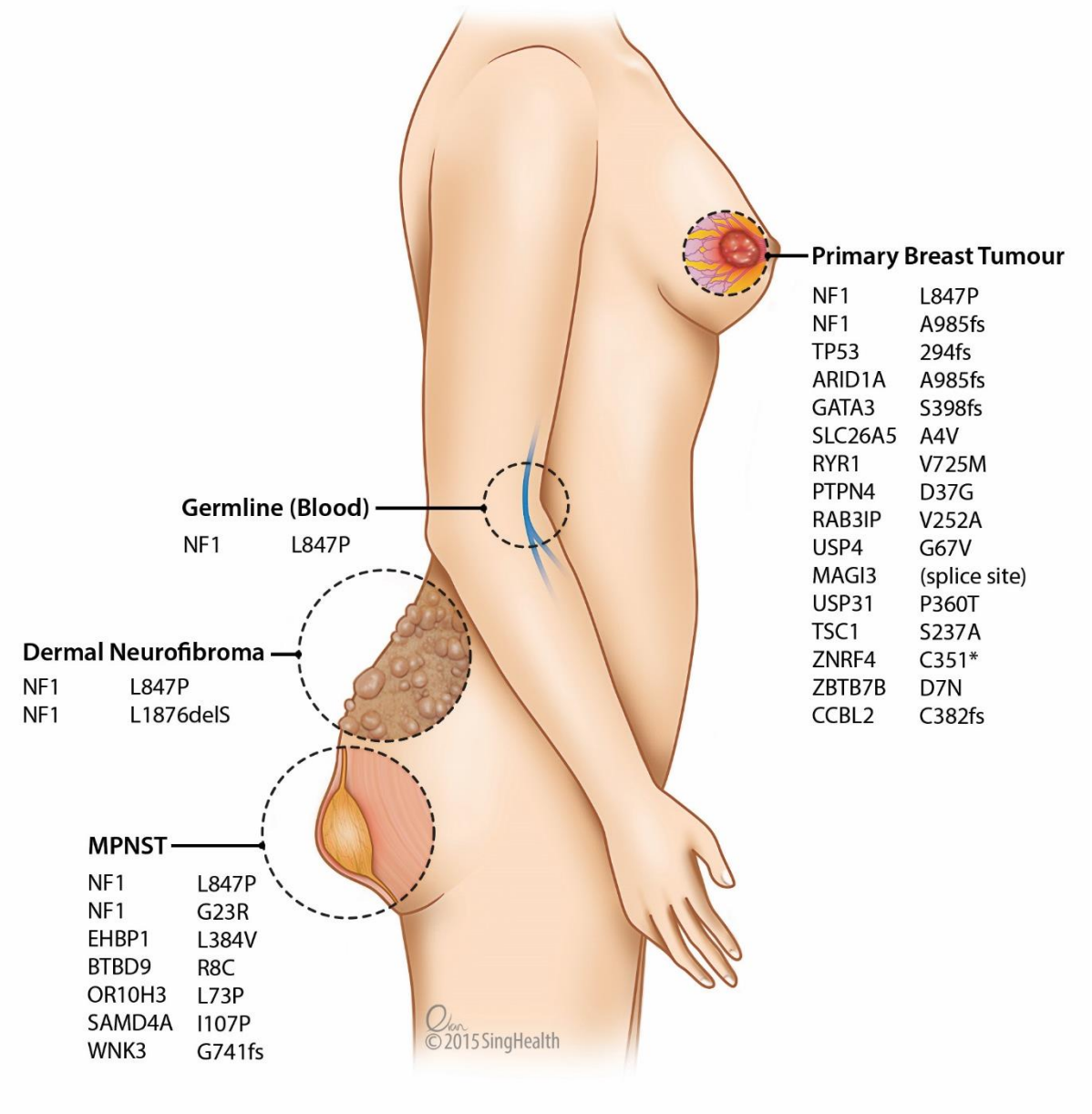

Figure 2.2: Somatic mutations in the three sequenced tumors.

Table 2.2: Mutation detected in normal, breast cancer, MPNST and dermal neurofibroma tissues from the same patient
* Mutation failed to validate by Sanger sequencing, but passed genome browser inspection

Gene Symbol	Tissue	Transcript ID	Strand	Chr	Pos	Genomic		cDNA		AA Change	Change Type	Sanger Validation	In Cosmic v52	Chromsomal Gain/Loss
NF1	blood (normal)	CCDS42292.1	+	chr17	29,556,173	g.chr17:29,556,173	T>C	c.2540	T>C	p.L847P	Missense	**Germline**		N.A.
NF1	breast cancer	CCDS42292.1	+	chr17	29,556,173	g.chr17:29,556,173	T>C	c.2540	T>C	p.L847P	Missense	**Germline**		N.A.
NF1	MPNST	CCDS42292.1	+	chr17	29,556,173	g.chr17:29,556,173	T>C	c.2540	T>C	p.L847P	Missense	**Germline**		N.A.
NF1	dermal neurofibroma	CCDS42292.1	+	chr17	29,556,173	g.chr17:29,556,173	T>C	c.2540	T>C	p.L847P	Missense	**Germline**		N.A.
NF1	breast cancer	CCDS42292.1	+	chr17	29,553,477	g.chr17:29,553,477	+C	c.2026	+C	p.P678Pfs	frameshift	**Somatic***	yes	Neg
NF1	MPNST	ENST00000444181.2	+	chr17	29,665,026	g.chr17:29,665,026	G>A	c.71	G>A	p.G23R	Missense	**Somatic**		N.A.
NF1	dermal neurofibroma	CCDS42292.1	+	chr17	29,657,329	g.chr17:29,657,329	-TCT	c.5625	-TCT	p.L1876del	In-frame	**Somatic**		Neg
ARID1A	breast cancer	CCDS44091.1	+	chr1	27,093,021	g.chr1:27,093,021	-G	c.2952	-G	p.A985fs	frameshift	**Somatic**		Neg
CCBL2	breast cancer	CCDS30767.1	-	chr1	89,408,741	g.chr1:89,408,741	-TA	c.1147	-TA	p.C382fs	frameshift	**Somatic**		Neg
GATA3	breast cancer	CCDS31143.1	+	chr10	8,115,845	g.chr10:8,115,845	-CA	c.1194	-CA	p.S398fs	frameshift	**Somatic**		Neg
MAGI3	breast cancer	CCDS44196.1	+	chr1	114,191,856	g.chr1:114,191,856	C>G	c.2156-3	C>G	-	possible 3' splice site	**Somatic**		Neg
PTPN4	breast cancer	CCDS2129.1	+	chr2	120,567,539	g.chr2:120,567,539	A>G	c.110	A>G	p.D37G	Missense	**Somatic**		Neg
RAB3IP	breast cancer	CCDS8993.1	+	chr12	70,188,943	g.chr12:70,188,943	T>C	c.755	T>C	p.V252A	Missense	**Somatic**		Neg
RYR1	breast cancer	CCDS33011.1	+	chr19	38,949,791	g.chr19:38,949,791	G>A	c.2173	G>A	p.V725M	Missense	**Somatic**		Neg
SLC26A5	breast cancer	CCDS5733.1	-	chr7	103,061,951	g.chr7:103,061,951	G>A	c.11	C>T	p.A4V	Missense	**Somatic**		Neg
TP53	breast cancer	CCDS11118.1	-	chr17	7,577,058	g.chr17:7,577,058	-C	c.880	-G	p.294fs	frameshift	**Somatic**	yes	Neg
TSC1	breast cancer	CCDS6956.1	-	chr9	135,796,778	g.chr9:135,796,778	A>C	c.709	T>G	p.S237A	Missense	**Somatic**		Neg
USP31	breast cancer	CCDS10607.1	-	chr16	23,116,773	g.chr16:23,116,773	G>T	c.1078	C>A	p.P360T	Missense	**Somatic**		Neg
USP4	breast cancer	CCDS2793.1	-	chr3	49,372,931	g.chr3:49,372,931	C>A	c.200	G>T	p.G67V	Missense	**Somatic**		Neg
ZBTB7B	breast cancer	CCDS1081.1	+	chr1	154,987,155	g.chr1:154,987,155	G>A	c.19	G>A	p.D7N	Missense	**Somatic**		Gain 1copy

ZNRF4	breast cancer	CCDS42475.1	+	chr19	5,456,555	g.chr19:5,456,555	C>A	c.1053	C>A	p.C351X	Nonsense	**Somatic**		Neg
BTBD9	MPNST	CCDS47418.1	-	chr6	38,565,849	g.chr6:38,565,849	G>A	c.22	C>T	p.R8C	Missense	**Somatic**		N.A.
EHBP1	MPNST	CCDS1872.1	+	chr2	63,101,527	g.chr2:63,101,527	T>G	c.1150	T>G	p.L384V	Missense	**Somatic**		N.A.
OR10H3	MPNST	CCDS12334.1	+	chr19	15,852,420	g.chr19:15,852,420	T>C	c.218	T>C	p.L73P	Missense	**Somatic**		N.A.
SAMD4A	MPNST	CCDS32084.2	+	chr14	55,168,905	g.chr14:55,168,905	A>T	c.322	A>T	p.I107F	Missense	**Somatic**		N.A.
WNK3	MPNST	CCDS35302.1	-	chrX	54,282,200	g.chrX:54,282,200	-C	c.2223	-G	p.G741fs	frameshift	**Somatic**		N.A.

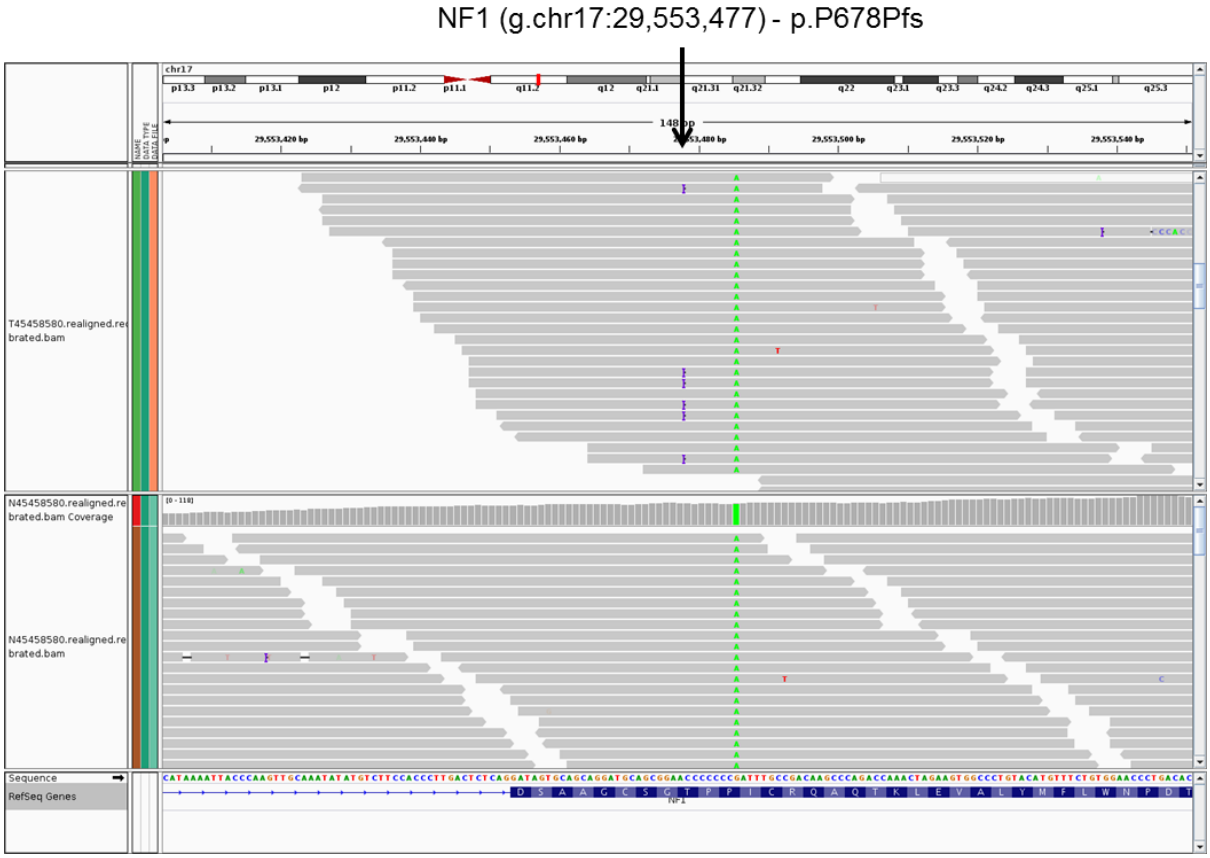

Figure 2.3: The sequencing reads as shown in the Integrated Genome Viewer software shows an insertion present (denoted with magenta ‡ mark) in the breast cancer that is not present in the germline (blood) sample.

Consistent with the fact that malignant tumors harbor more somatic mutations, the breast cancer and MPNST harbored 15 and 6 somatic mutations, respectively (Figure 2.2, Table 2.2). In contrast, only one somatic mutation was detected in the dermal neurofibroma, a benign tumor (Figure 2.2). Interestingly, this in-frame deletion of 3 nucleotides resulted in a loss of leucine at amino acid position 1876 in the *NF1* gene (Table 2.2). Different somatic *NF1* mutations (frameshift insertion in breast cancer (p.A985fs), missense mutation in MPNST (p.G23R**)**, and inframe deletion in dermal neurofibroma (p.L1876del-Inf)) were detected in all three tumors investigated; suggesting the importance of second-hit aberration in *NF1* for tumorigenesis.

Several important and interesting mutations were identified in the 2 malignant tumors. The breast tumor harbored somatic mutations in *TP53*, *GATA3* and *ARID1A*, which are commonly mutated or lost in breast cancer (Figure 2.2). Mutations in other cancer-

associated genes such as *MAGI3*, *TSC1*, *PTPN4*, *RAB3IP* and *RYR1* were also identified. Aberrant protein degradation may be important in this cancer, as 3 of the 14 mutated genes (*USP4*, *USP31* and *ZNRF4*) in the breast cancer are involved in the ubiquitin-proteasome pathway. In the MPNST, apart from the *NF1* mutation, other mutations in cancer-associated genes such as *EHBP1* and *WNK3* were identified.

Extensive copy number alterations detected in breast cancer and MPNST

To determine the extent of chromosomal aberrations in the breast cancer, the MPNST and the dermal neurofibroma, we subjected the sequence data to our in-house modified ASCAT analysis. Extensive chromosomal aberrations were observed in the breast cancer and the MPNST samples. Unlike the profiles seen in the breast cancer and MPNST, the benign dermal neurofibroma genome is “silent”, indicating that the neurofibroma genome is highly stable (Figure 2.4).

Gains and losses in several cancer-related genes were identified in the breast cancer and MPNST samples through ASCAT analysis (Supplementary Table S2.1). Consistent with a previous report on chromosomal aberrations in MPNST, losses were more common than gains in the MPNST on ASCAT analysis [18]. Homozygous losses were found in *CDKN2A* and *CDKN2B,* which play important roles in cell cycle control, and in *ARID4B*, a chromatin remodeling gene. Heterozygous losses were observed in *TP53* and *EED*, a component of the Polycomb repressive complex 2 (PRC2), which is reported to be frequently altered in a recent exome sequencing study on MPNSTs [14].

Potential oncogenes amplified in the breast cancer include *GREB1*, *NRXN1*, *MGAT5, PKP4*, *DAPL1*, *ITGB6* and *RBMS1*, which may be implicated in carcinogenesis, proliferation and invasion. A focal amplification of around 3 Mb in chromosome 2, containing the *FXBO11* and *MSH6* genes, was estimated to contain over 20 copies of the affected genes. We observed heterozygous loss of several cancer-associated genes, including the *CTNNA1* (catenin (cadherin-associated protein), alpha 1) and *APC* tumour suppressor genes in the breast cancer. With the available DNA, we further confirmed our findings in the breast cancer and dermal neurofibroma using the Cytoscan platform from Affymetrix.

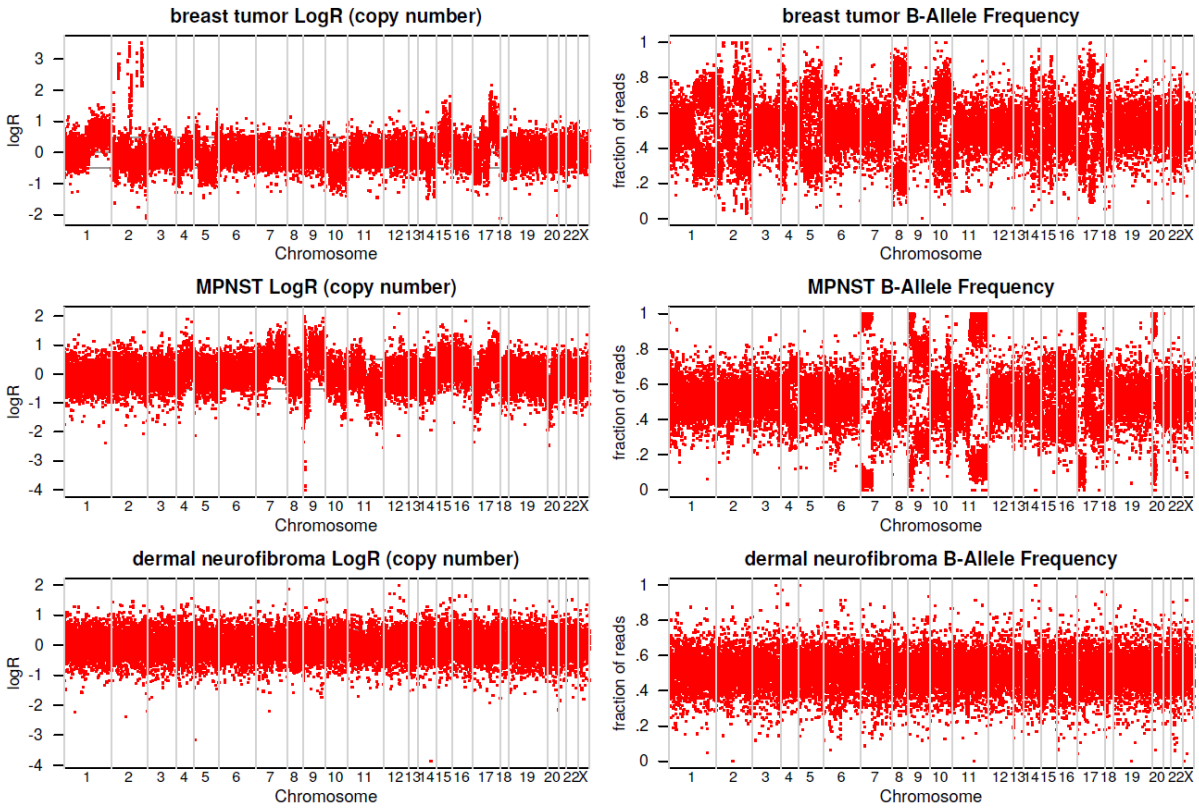

Figure 2.4: Analysis of copy number alterations in breast cancer (A), malignant peripheral nerve sheath tumor (MPNST) (B) and dermal neurofibroma (C), based on paired tumor and nontumor DNA analysis of exome sequencing data.

Each red dot represents a genomic coordinate that was heterozygous in the germline sample. The breast cancer shows the most chromosomal rearrangements while the dermal neurofibroma shows none. Regions of allelic imbalance that also show a decrease in Log R typically represent Loss-of-Heterozygosity (LOH); regions of allelic imbalance with no change in Log R show Copy-Number Neutral LOH, and regions of allelic imbalance that correspond with increased Log R correspond to either focal amplifications (for example, several loci in chromosome 2 of the breast cancer) or large-scale amplifications. The changes in Log R and allele frequency observed across multiple chromosomes in breast cancer and MPNST indicate allelic imbalance or copy number alterations in these samples, but not in the dermal neurofibroma.

Using Cytoscan, copies gained were observed in a large region of chromosome 2 (p25.1, p24.2, p21, q14.2, q21.2, q23.3, q24.3, q33.1, q36.1, q36.2, q36.3 and q37.2), chromosome 4 (p15.2), chromosome 15 (q22.2 and q24.2) and chromosome 17 (q12 and q21.32), while loss of heterozygosity was observed in chromosomes 2, 4, 5, 10, 14, 17 and 18 in the breast cancer genome (Figure 2.5 and Supplementary Table S2.2).

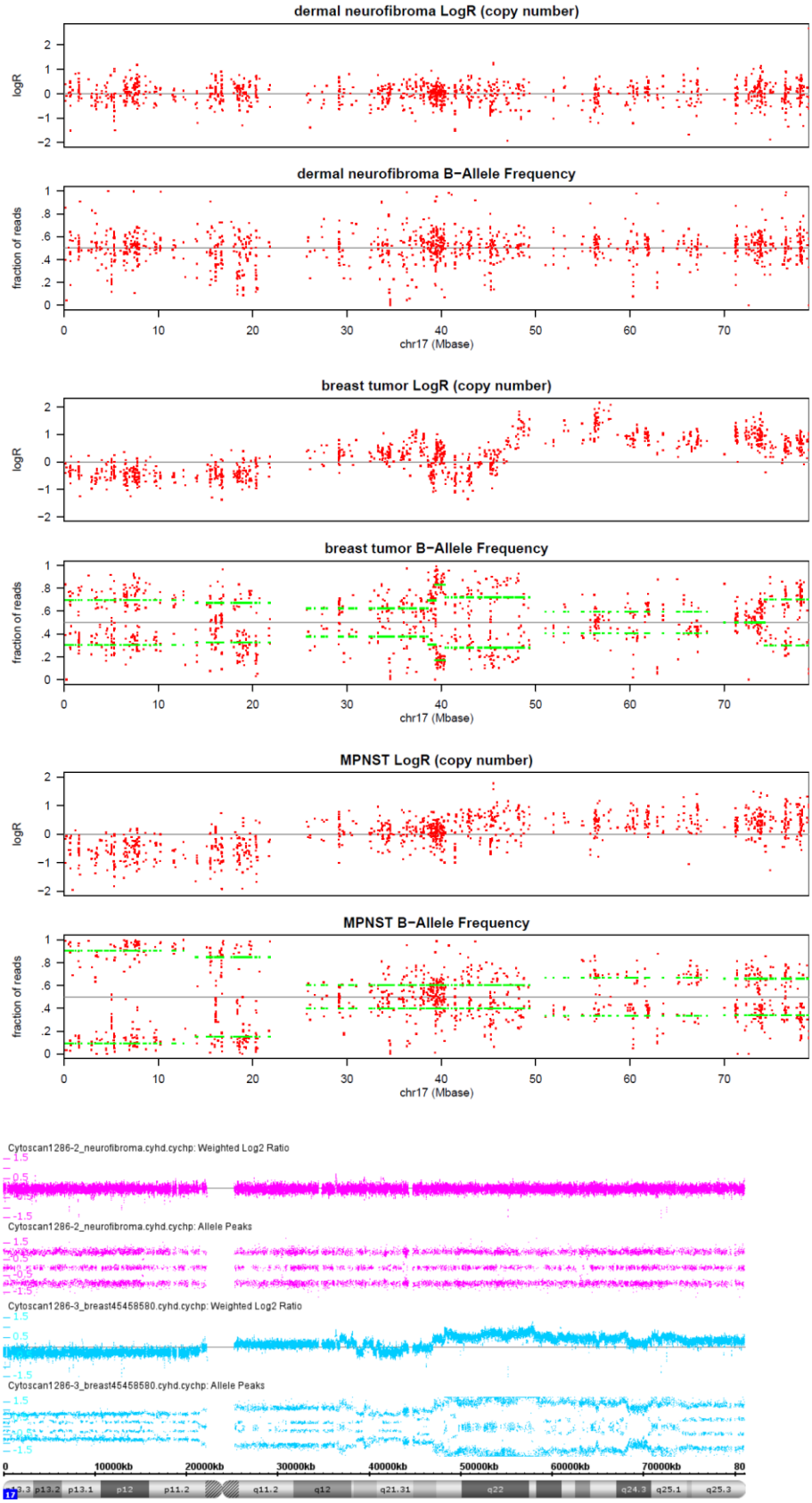

Figure 2.5: both exome sequencing and Cytoscan HD SNP Array show LOH in chr17p for the breast cancer (both platforms) and the MPNST (only exome sequencing available). LOH is indicated by a drop in copy number coinciding with allelic imbalance.

Interestingly, we have identified loss of chromosome 6q22.33 (Figure 2.6) and gain of chromosome 14q32.33 in the DNA from the patient's blood, breast cancer and dermal neurofibroma suggesting germline chromosomal aberrations. Two putative tumor suppressor genes, *PTPRK* and *LAMA2,* are among the 10 genes identified in the loss region of chromosome 6; these germline chromosomal alterations might predispose the patient to cancer.

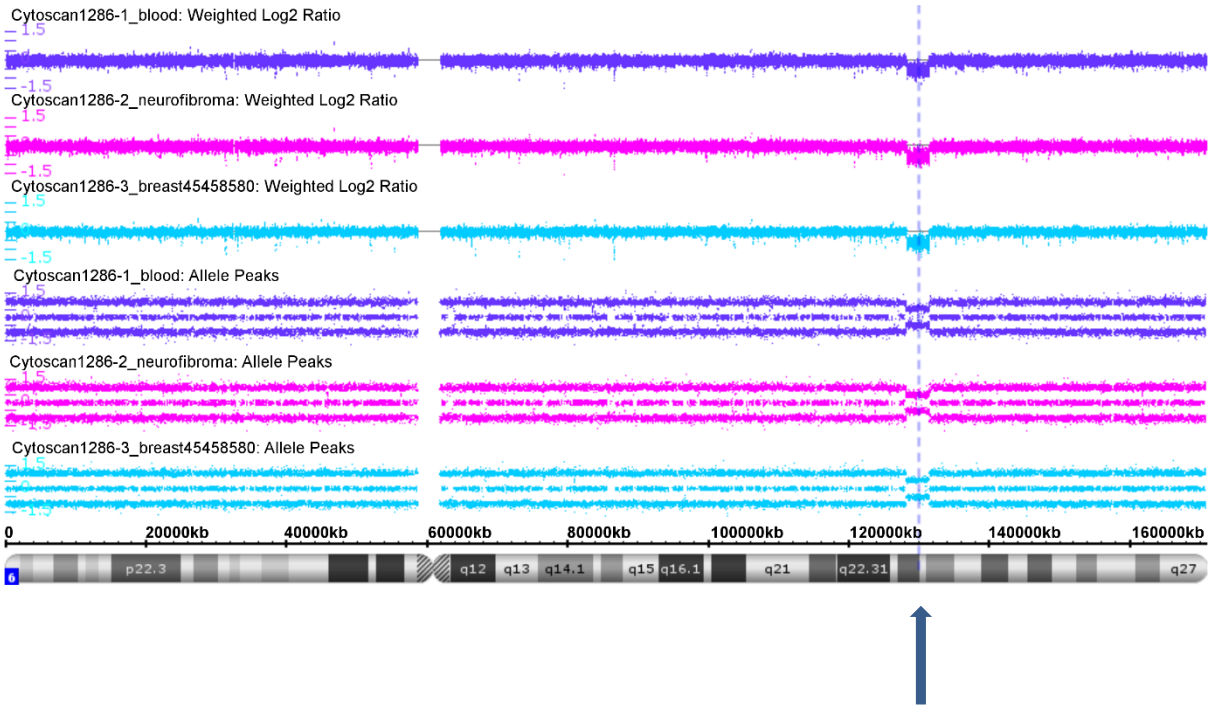

Figure 2.6: Cytoscan HD SNP Array analysis reveals a germline Loss-of-heterozygosity event of around 3.3 Mbases at chr6q22.33 (indicated by dottedline) which harbor *PTPRK* and *LAMA2*, in blood, breast cancer, and dermal neurofibroma.

2.5 Discussion

Whole exome sequencing of the blood, breast cancer, MPNST and dermal neurofibroma from this patient has provided invaluable insight into the somatic anomalies in this tumour predisposition syndrome. A striking feature is the finding of second-hit *NF1* mutation at different sites of this gene in all the tumours sequenced, indicating that second-hit mutation of this tumour suppressor gene may be a critical event in pathogenesis. *NF1* aberrations can potentially lead to activation of the Ras, MAP kinase and PI3K-mTOR pathways, resulting in proliferation of tumour cells [3,19]. This is consistent with the findings of a previous study which reported different somatic *NF1* alterations in multiple benign neurofibromas and a MPNST obtained post-mortem from an NF1 patient [20]. Denaturing High Performance Liquid Chromatography (dHPLC), microsatellite analysis using RFLP markers and multiplex ligation probe amplification (MLPA) for the *NF1* gene were performed in that study [20]. With whole exome sequencing, we have found that each tumour from the same individual also has a distinct set of genes mutated. The different clonal origins indicate that each tumour arises from independent somatic events in the background of a heterozygous germline *NF1* mutation.

Incidentally, we have also discovered germline loss, resulting in loss-of-heterozygosity of two potential tumour suppressor genes, *PTPRK* and *LAMA2*, both located at chromosome 6q22.33. *PTPRK* appears to be a negative regulator of adhesion, invasion, migration, and proliferation in various tumour types, including breast and colorectal cancers, gliomas, lymphoma and melanoma cells. It may play a role in inhibition of Akt, EGFR and beta-catenin signaling [21-25]. *LAMA2* encodes an extracellular matrix protein. Reduced *LAMA2* expression in hepatocellular carcinomas has been linked to a proliferative signature with poorer survival outcomes; hypermethylation of *LAMA2* has also been reported in colorectal carcinomas [26,27]. While germline loss of *PTPRK* and *LAMA2* may have been reported in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) in a small number of individuals, the context in which they occur are not clear. We hypothesise that heterozygous loss of this region of chromosome 6q may increase tumour predisposition further in this NF1 patient.

The mutation landscape of breast cancer is highly heterogeneous. In this case of NF1-associated breast cancer, mutations in the *TP53*, *TSC1* and *MAGI3* (PI3K/Akt/mTOR pathway) tumour suppressors are likely to cooperate with *NF1* in carcinogenesis. In addition, alterations of genes critical in other pathways, such as *ARID1A* (SWI/SNF chromatin remodeling), *GATA3* (differentiation of luminal breast cells), *PTPN4* (anti-apoptosis), *RYR1* (control of cellular proliferation) and *USP4, USP31, ZNRF4* (ubiquitin-proteasome pathway) may be implicated in pathogenesis. The multiple copy number alterations detected also result in genomic instability. Gains in some of the genes involved in invasive/migratory properties (*MGAT5, PKP4, ITGB6*), cell cycle progression and regulation of apoptosis (*RBMS1*), and angiogenesis (*NRXN1*) may be involved in tumor development and progression. Amplification of *GREB1*, an early response gene in the estrogen receptor-regulated pathway, may play a role in this estrogen receptor positive tumour.

The mechanism of pathogenesis for the MPNST is likely related to second-hit inactivation of *NF1*, together with mutations in other genes, such as *WNK3* which plays a role in the increase of cell survival in a caspase-3-dependent pathway, and *EHBP1* which has been implicated in endocytic trafficking. A single nucleotide polymorphism in *EHBP1* has been associated with an aggressive form of prostate cancer; it may be implicated in carcinogenesis or cell survival [28]. Somatic loss of critical tumour suppressors including *CDKN2A*, *CDKN2B*, *TP53* and *EED* are also likely to contribute to tumorigenesis. Aberrations in *EED* and *SUZ12* occur frequently in sporadic, NF1-associated and radiotherapy-associated MPNSTs [14-16]. EED and SUZ12 are core subunits of PRC2; the resulting PRC2 inactivation can lead to loss of trimethylation at lysine 27 of histone H3 (H3K27me3), and increased H3K27 acetylation which recruits bromodomain proteins and transcription factors to promote tumour growth [14,16].

The low mutation burden and lack of copy number changes in the dermal neurofibroma is consistent with the benign nature of this tumour. Each neurofibroma consists of a heterogeneous collection of hyperproliferative Schwann cells, as well as fibroblasts, perineural cells and mast cells [20]. Although the second-hit somatic *NF1* mutation in the Schwann cells may trigger the formation of a neurofibroma in an *NF1*-haploinsufficient microenvironment, the precise molecular interactions among the different cells in the development of neurofibromas remain poorly understood.

Challenges remain in the detection of aberrations in *NF1* gene. *NF1* is one of the largest human genes, with 60 exons and 350kB of genomic DNA. While next generation sequencing platforms may facilitate the simultaneous sequencing of the various exons, it may not capture intronic splicing mutations; analysis to detect aberrations and validation of mutations can pose a major challenge. Design of primers to validate the mutations detected with next generation sequencing can be complicated and not feasible at certain sites along the large gene, due to the presence of multiple pseudogenes in the human genome[8].

In summary, second-hit inactivation of *NF1* appears to be a common feature of various tumours in NF1 syndrome. However, the complexity of the pathogenesis of various tumours remains to be elucidated. Besides loss of *NF1* function, additional aberrations in other important cancer related genes involving various pathways lead to the development of specific tumours. Further investigations on additional tumour specimens from more NF1 patients will improve our understanding of the mechanisms of pathogenesis in this tumor predisposition syndrome.

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2.7 Supplementary data

Supplementary Table S2.1: the estimated copy number for various cancer-related genes, based on comparing the read-depth coverage of the tumour sample to the matched non-malignant sample. Segmentation and copy-number modelling were performed with the ASCAT software.

Supplementary Table S2.2: the estimated copy number for genes with gains or losses as detected by the Chromosome Analysis Suite software from Affymetrix for Cytoscan HD Array data.

Chapter 3

Breast cancer in women with neurofibromatosis type 1 (NF1) – a comprehensive case series with molecular insights into its aggressive phenotype

3.1 Abstract

This study aimed to improve the understanding of NF1-associated breast cancer, given the increased risk of breast cancer in this tumour predisposition syndrome and the limited data. We identified 18 women with NF1 and breast cancer at our institution. Clinical and pathologic characteristics of NF1-associated breast cancers were compared with 7132 breast cancers in patients without NF1 from our institutional database.

Next generation sequencing was performed on DNA from the blood and breast cancer specimens available from the 18 NF1 patients. When this did not detect germ-line *NF1* mutations, multiplex ligation-dependent probe amplification (MLPA) was used to identify if there were complete/partial deletions or duplications of the *NF1* gene. Expression of neurofibromin in the NF1-associated breast cancers was evaluated using immunohistochemistry.

There was a higher frequency of grade 3 (83.3% vs 45.4%, p=0.005), oestrogen receptor (ER) negative (66.7% vs 26.3%, p<0.001) and human epidermal growth factor receptor 2 (HER2) positive (66.7% vs 23.4%, p<0.001) tumours among NF1 patients compared to non-NF1 breast cancers. Overall survival was inferior in NF1 patients in multivariable analysis (hazard ratio 2.25, 95% CI, 1.11 to 4.60; p = 0.025). Apart from germline *NF1* mutations (11/16 detected; 69%), somatic mutations in *TP53* (8/10; 80%), second-hit *NF1* (2/10; 20%), *KMT2C* (4/10; 40%), *KMT2D* (2/10; 20%), and *PIK3CA* (2/10; 20%) were observed. Immunohistochemical expression of neurofibromin was seen in the nuclei and/or cytoplasm of all specimens, but without any consistent pattern in its intensity or extent. This comprehensive series of NF1-associated breast cancers suggests that their aggressive features are related to germline *NF1* mutations in cooperation with somatic mutations in *TP53, KMT2C* and other genes.

3.2 Introduction

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen disease, is a relatively common genetic disorder with a prevalence of 1 in 2,500 to 3,000 individuals worldwide. While the condition is inherited in an autosomal dominant fashion, about half of the cases are sporadic, that is, without any family history [1,2]. Characterized by the development of multiple neurofibromas, café-au-lait spots and Lisch nodules, individuals with NF1 are at increased risk of developing benign and malignant tumours in addition to a range of abnormalities in the neurological, cardiovascular and musculoskeletal systems [3]. The average life expectancy among individuals with NF1 is reduced by 10-15 years, with cancer being the most common cause of death [4].

This tumour predisposition disorder is related to germline aberrations in the NF1 gene on the long arm of chromosome 17. Its product neurofibromin has a central region which has marked structural and sequence similarity to ras-guanosine-triphosphate(GTP)ase activation proteins (GAPs). GAPs inactivate Ras by accelerating the conversion of active Ras- guanosine triphosphate (GTP) to its inactive guanosine diphosphate (GDP)-bound form [5,6]. The downregulation of Ras by neurofibromin prevents the downstream activation of the mitogen-activated protein kinase (MAPK) and PI3K/Akt/mTOR (mammalian target of rapamycin) cell proliferation and differentiation pathways. In addition, other mechanisms of tumour suppression include promotion of apoptosis, regulation of cell adhesion and motility as well as suppression of heat shock protein and epithelial mesenchymal transition [3].

The classic NF1-associated tumours include malignant peripheral nerve sheath tumours (MPNSTs), optic pathway gliomas, rhabdomyosarcomas, neuroblastomas, juvenile myelomonocytic leukaemias, gastrointestinal stromal tumour (GIST) and phaeochromocytomas [7]. More recently, an increased risk of breast cancer has also been reported. In the first such study reported by Sharif et al in 2007, within a cohort of 304 women with NF1 in north-western region of England, there were 14 cases of breast cancers over the follow-up period from 1975-2005. The overall standardized incidence ratio (SIR) was 3.5 (95% CI 1.9-5.9), but the SIR was as high as 4.9 (95% CI 2.4-8.8) for women aged <50 years [8]. In a subsequent study from the metropolitan Detroit area, 9 out of 76 women with NF1 developed breast cancer over the period

from 1990 to 2009. The overall SIR was 5.2 (95% CI 2.4-9.8), but this relative increase was even higher for women with NF1 under the age of 50 years, with SIR 8.8 (95% CI 3.2-19.2) [9]. Subsequent studies by various groups have generally supported these findings, namely an increased risk of breast cancer among women with NF1 compared to the general population, with an even higher risk among younger women [10-12]. The largest study identified 58 cases of breast cancer among 3672 women with diagnosis of NF1 admitted to National Health Service hospitals in England over the period 1999-2011. The relative risk compared to the control cohort decreased with increasing age: 6.5 among women aged 30-39 years, to 4.4 in the 40-49 year age group, 2.6 in the 50-59 age group, 1.9 in the 60-69 year age group, and 0.8 in the 70-79 year age group [11]. Breast cancer is now recognized as one of the NF1-related malignancies, hence NF1-associated breast cancer is not just a random set of sporadic breast cancers. *NF1* has been unequivocally identified as a breast cancer susceptibility gene which confers moderate risk of breast cancer [13].

The clinical and molecular data on NF1-associated breast cancer is currently limited. We previously presented our preliminary findings on the aggressive nature and high frequency of HER2 overexpression of breast cancers in women with NF1 [14]. We hypothesized that compared to sporadic breast cancers, NF1-associated breast cancers are more aggressive with a higher risk of poor prognostic features as well as inferior outcomes. In this study, we aimed to further characterise the molecular profile of NF1-associated breast cancers. Clinical and pathologic characteristics of NF1-associated breast cancers were compared with non-NF1 breast cancers at our institution. Next generation sequencing was performed on DNA from blood and breast cancer specimens available, and the expression of neurofibromin in the breast tumours was evaluated using immunohistochemistry.

3.3 Methods

Patients

Patients with both NF1 and breast cancer were identified retrospectively from hospital records as well as prospectively by the candidate YSY when managed at National Cancer Centre Singapore (NCCS) and Singapore General Hospital (SGH). All women

had histologically proven breast cancer and fulfilled at least 2 of the 7 criteria developed by the NIH Consensus Conference for clinical diagnosis of NF1. Each patient, with the exception of deceased patients, provided written informed consent prior to study entry. The study design was conceived by the candidate, who also obtained funding for the study. The study protocol was approved by the Singapore Health Services (SingHealth) institutional review board.

Details on patient demographics, tumour grade, stage, receptor status and clinical outcome were evaluated by the candidate from medical records; majority of the patients were managed by the candidate. The pathological characteristics of NF1-associated breast cancers were compared to 7132 breast cancers in patients without NF1 captured in our breast cancer tumour board database over a similar period from 2001-2016, with a focus on grade and HER2 positivity rate. For patients with metachronous bilateral breast cancer, only the first cancer was included. For patients with synchronous bilateral breast cancer, the tumour with the higher stage was included. If both tumours were the same stage, the patient was excluded from the analysis.

Oestrogen receptor (ER) and progesterone receptor (PR) status was considered positive with a minimum of 1% of cells staining positive [15]. Human epidermal growth factor receptor 2 (HER2) positivity was defined as 3+ immunohistochemical staining in more than 10% of cells, or HER2 positivity on fluorescence *in situ* hybridization (FISH) testing as per latest guidelines [16].

Statistical Analyses

Tumour characteristics were compared between NF1 and non-NF1 (control) breast cancers using Fisher's exact test (for categorical variables) and Mann-Whitney U test (for continuous variables). Overall survival (OS) was defined as time from diagnosis to death from any cause. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. Median follow-up time was estimated using the reverse Kaplan-Meier method. Univariable and multivariable analyses were performed using the Cox regression model. The proportional hazards assumption was assessed graphically by plotting the log-log survival curves. All analyses were

performed in Stata (version 14, StataCorp, Texas, USA). Two-sided *p*-values less than 0.05 were considered statistically significant.

Targeted sequencing

Germline DNA was extracted from peripheral blood while tumour DNA was obtained from fresh, frozen samples or formalin-fixed paraffin-embedded (FFPE) blocks containing at least 60% of tumour nuclei using QiaAmp DNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNA was extracted from the blood and the fresh frozen specimens by the candidate and the team at Laboratory of Molecular Oncology, Division of Medical Sciences, National Cancer Centre Singapore while DNA from FFPE tumour specimens was extracted by the Singapore General Hospital Pathology Department, who also assisted with assessment of tumour content and marking of the tumour specimens.

Sequencing libraries were prepared from genomic DNA using the Kapa Hyper Prep kit, and captured using the IDT Xgen 127-gene Pan-cancer panel, or using the SureSelect XT2 target enrichment kit (Agilent, Santa Clara, CA, USA) for a customised panel of 338 genes as previously reported [17] (Supplementary Table S1 for gene panels), all following manufacturers' instructions. Quality control and library preparation were performed by collaborators at the Laboratory of Molecular Oncology, Division of Medical Sciences, National Cancer Centre Singapore for the specimens captured with the SureSelect XT2 panel, and by collaborators at Genome Institute of Singapore, A*STAR, Singapore, for the IDT Xgen 127-gene Pan-cancer panel. Libraries were pooled and sequenced at sequencing facilities in A*STAR, Singapore with a 150bp paired-end run on an Illumina Miseq for the samples captured with the IDT Xgen panel, and on the Illumina Hiseq 4000, with 100bp paired end reads for the samples captured with the SureSelect XT2 panel. Whole exome sequencing was performed on one of the matched tumour-blood specimens as published previously [18].

MLPA

Blood specimens which tested negative for *NF1* mutations were subjected to multiplex ligation-dependent amplification (MLPA) to detect single and multi-exon *NF1* gene copy number variations, using the test kits (SALSA MLPA P122 *NF1*-area probemix) and SALSA MLPA P082 *NF1*-mix2 probemix (MRC Holland, Amsterdam, Netherlands)

[19] . MLPA was performed by the candidate and the collaborators at the Laboratory of Molecular Oncology, Division of Medical Sciences, National Cancer Centre Singapore As previously described [17], DNA fragment analysis was performed on the ABI 3130 Genetic Analyzer (ABI-Life Technologies, Thermo Fisher Scientific Corporation, MA, USA), and analysed with the Coffalyser freeware v.131123.1303 (MRC-Holland).

Sequencing Data Analysis

Sequencing reads were aligned to the human reference genome hg19 using BWA tool, and PCR duplicates were removed using Picard tool (http://broadinstitute.github.io/picard/). SNPs and indels were called using the Genome Analysis Toolkit (GATK) HaplotypeCaller algorithm [20]. After examining the sequencing coverage, samples with mean read depth coverage of < 50x were excluded from further analysis. Manual curation using the Integrative Genomics Viewer (IGV) was also conducted. Variants detected were annotated using the ANNOVAR tool [21] and those identified with minor allele frequency (MAF) > 1% in all populations from publicly available databases such as the 1000 Genomes project [22] and ExAC [23] were excluded. Variants were considered pathogenic if they were frameshift insertions or deletions, stop gain (nonsense) or splice site changes. For missense variants, pathogenicity was predicted with the use of the following tools: SIFT [24], PolyPhen-2 [25], MutationTaster [26], MutationAssessor [27] and CADD [28]. A pathogenicity score was calculated using all five functional prediction tools, and considered pathogenic if at least one of the five tools classified the variants as being deleterious. Pipeline analysis was performed by the collaborating bioinformatician from the Laboratory of Molecular Oncology at the Division of Medical Sciences, with the candidate and the co-supervisor Associate Professor Ann Lee for the interpretation of the results.

Immunohistochemistry

Collaborators from the Pathology department at Singapore General Hospital were involved in this part of the study. The sections (4µm) were cut from tissue blocks, mounted on Leica BOND Plus slides (Leica Biosystems, Richmond, IL, USA) and dried on heating bench for 20 minutes. Immunohistochemical procedure was performed using the Leica Bond Autostainer (Leica Biosystems, Mt. Waverley, VIC, Australia).

The sections were deparaffinised and pretreated using bond dewax reagents and Bond Epitope Retrieval Solution 2 (Leica Biosystems, Newcastle Upon Tyne, UK) of pH 8.9 to 9.1. Endogenous peroxidase activity was blocked using hydrogen peroxide for 5 minutes followed by *NF1* primary antibody (ab30325, 1:300 dilution; Abcam, Cambridge, UK) incubation for 20 minutes. The sections were then treated with post primary and polymer reagents followed by mixed DAB refine reagent. The detection system used was Bond Polymer Refine (Leica Biosystems, Newcastle Upon Tyne, UK). The sections were counterstained with haematoxylin and the slides were unloaded from the system, then dehydrated and mounted in DPX (CellPath, Newtown, UK). For immunoscoring, the staining intensity (nil, weak (1+), moderate (2+), strong (3+)) and the proportion of tumour cells stained in the nuclei as well as the cytoplasm were recorded. Tonsillar tissue, which is known to express low neurofibromin protein within the germinal centres (https://www.proteinatlas.org/ENSG00000196712-NF1/tissue/tonsil), was used as positive control.

3.4 Results

Clinicopathological characteristics of NF1-associated breast cancers

We identified 18 patients with NF1 and breast cancer managed at our institutions from 2000 to 2016 (Table 3.1). Median age of breast cancer diagnosis was 49.5 years (range 30-75 years). All 18 women had invasive ductal carcinoma but one of them was also diagnosed with an incidental synchronous contralateral invasive lobular carcinoma. Seven of the patients (38.9%) had stage 3 or 4 breast cancer at diagnosis. Fifteen out of 18 tumours or 83.3% were grade 3, compared to 45.4% among our non-NF1 controls (p=0.005) (Table 3.2). The majority (12 of 18, 66.7%) of the NF1-associated breast cancers were HER2 positive, compared to 23.4% in controls (p<0.001). Of the remaining NFI-associated tumours, four were triple negative, and two were hormone receptor positive and HER2 negative. NF1-associated cancers were also more likely to be oestrogen receptor (ER) negative (66.7% versus 26.3%; p<0.001) and progesterone receptor (PR) negative (66.7% versus 37.8%; p=0.015) than controls.

To date, 6 out of 16 patients with stage 1-3 breast cancer (37.5%) have relapsed, all

of whom had HER2+ tumours. Five-year overall survival (OS) was 69.6% (95% CI, 41.0 to 86.4%) in the NF1 group, and 84.5% (95% CI, 83.4 to 85.5%) in the control group of non-NF1 breast cancers (p = 0.017). As tumour grade, stage and subtype were predictive of OS in univariate analysis (Table 3.3) but did not adhere to the proportional hazards assumption, multivariable analysis was performed using a Cox model stratified on these variables. Age at diagnosis was included as a covariate in the model. The resulting hazard ratio for NF1 vs. controls was 2.25 (95% CI, 1.11 to 4.60; p = 0.025) (Table 3.4).

Table 3.1: Clinicopathologic characteristics of NF1-associated breast cancers

Patient ID	Age at diagnosis (years)	Ethnicity	Stage at diagnosis	Grade	Histology	ER	PR	HER2	Subtype	*NF1* Germline Mutation	Family History of NF1	Other Tumours	Time from Diagnosis to Relapse (months)	Systemic Therapy given for Breast Cancer (according to sequence of administration)	Overall Survival from Diagnosis (months)
1	30	Chinese	2 (T1N1M0)	3	IDC	+	+	+	HER2	NE	+	Nil	61	Adjuvant cyclophosphamide, methotrexate and 5-fluorouracil, tamoxifen; palliative goserelin and letrozole, 5-fluorouracil, epirubicin and cyclophosphamide, weekly paclitaxel.	92
2	32	Chinese	2 (T2N0M0)	3	IDC	-	-	-	TNBC	ND	+	Nil	NA	Adjuvant doxorubicin and cyclophosphamide	NA
3	39	Chinese	2 (T2N1M0)	3	IDC	+	+	+	HER2	p.L847P	+	MPNST (cause of death)	NA	Adjuvant doxorubicin cyclophosphamide, followed by paclitaxel with trastuzumab, maintenance trastuzumab and tamoxifen	93
4	42	Malay	3 (T3N1M0)	3	IDC	-	-	+	HER2	p.1446_1451del; non-frameshift deletion	-	Nil	19	Neoadjuvant doxorubicin cyclophosphamide, followed by paclitaxel chemotherapy with trastuzumab, maintenance trastuzumab; palliative capecitabine and lapatinib, vinorelbine and trastuzumab	22

5	44	Chinese	2 (T1N1M0)	3	IDC	-	-	+	HER2	p.L847P	-	Nil	58	Adjuvant doxorubicin cyclophosphamide; palliative docetaxel and trastuzumab, capecitabine and lapatinib, vinorelbine and trastuzumab, Gemcitabine and carboplatin, panobinostat and everolimus (clinical trial), Ixabepilone TDM-1, PI3K inhibitor (clinical trial)	125
6	44	Indian	1 (T1N0M0)	3	IDC	-	-	+	HER2	p.Y489C	-	Nil	24	Declined adjuvant systemic treatment initially, but after local recurrence, received doxorubicin cyclophosphamide, followed by paclitaxel with trastuzumab, maintenance trastuzumab	NA
7	45	Chinese	2 (T2N0M0)	3	IDC	+	+	-	Luminal	p.Q2686fs frameshift deletion	+	Nil	NA	Adjuvant docetaxel cyclophosphamide, tamoxifen	NA
8	48	Chinese	3 (T3N0M0)	2	IDC	-	-	+	HER2	ND	+	Nil	NA	Neoadjuvant doxorubicin cyclophosphamide, followed by paclitaxel with trastuzumab, maintenance trastuzumab	NA
9	49	Malay	4 (T4N3M1)	3	IDC	+	-	+	HER2	ND	-	Nil	NA	Palliative paclitaxel chemotherapy with trastuzumab,	5

10	50	Chinese	3 (T4N1M0)	3	IDC	-	+	+	HER2	NE	-	Nil	12	Neoadjuvant doxorubicin cyclophosphamide, followed by paclitaxel, maintenance trastuzumab and letrozole; palliative vinorelbine and trastuzumab, capecitabine and trastuzumab, cyclophosphamide methotrexate	29
11	52	Chinese	2 (T2N2M0)	2	IDC	+	+	-	Luminal	p.T2284fs frameshift deletion	+	Nil	NA	Adjuvant doxorubicin cyclophosphamide, followed by paclitaxel, letrozole	NA
12	54	Chinese	1 (T1N0M0)	3	IDC	-	-	+	HER2	ND	+	Nil	NA	Nil	NA
13	58	Malay	2 (T2N0M0)	3	IDC	-	-	+	HER2	p.Q1447H	+	GIST	NA	Adjuvant doxorubicin cyclophosphamide, followed by maintenance trastuzumab	NA
14	60	Chinese	3 (T3N1M0)	3	IDC	-	-	-	TNBC	p.R461X	-	Nil	NA	Adjuvant doxorubicin cyclophosphamide, followed by paclitaxel	NA
15	62	Chinese	3 (T2N2M0)	3	IDC	-	-	-	TNBC	ND	+	Nil	NA	Adjuvant doxorubicin cyclophosphamide, followed by paclitaxel	NA
16	62	Chinese	4 (T4N1M1)	3	IDC	-	-	+	HER2	p.Y2285X	-	Nil	NA	Palliative paclitaxel with trastuzumab,	2
17	64	Indian	2 (T2N1M0)	2	IDC	+	+	+	HER2	p.S2093fs frameshift deletion	-	Phaeochromocytoma	29	Adjuvant doxorubicin cyclophosphamide, followed by maintenance	56

														trastuzumab, tamoxifen, anastrozole. palliative paclitaxel and trastuzumab, capecitabine and lapatinib	
18	74	Chinese	2 (T2N0M0)	3	IDC	-	-	-	TNBC	p.Q2245X; stop gain	-	GIST, oncocytoma	NA	Nil	NA

Abbreviations

IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; TNBC: triple negative breast cancer; Luminal: ER/PR+HER2-; HER2: HER2+ regardless of ER/PR; NE: not evaluated; ND: not detected; NA: not applicable (eg patient has not relapsed or died.).

Table 3.2: Comparison of NF1-associated and non-NF1 (control) breast cancers.

	Controls, *n* (%)[a]	NF1-associated, *n* (%)[a]	*p*-value[b]
Histological subtype			
Ductal	6189 (86.8)	18 (100)	-
Lobular	366 (5.1)	0	
Other	577 (8.1)	0	
Age at diagnosis, years			
Median (range)	54 (21 – 97)	49.5 (30 – 75)	0.138
Unknown	2	0	
Tumour grade			
1	1022 (15.2)	0	0.005
2	2637 (39.3)	3 (16.7)	
3	3043 (45.4)	15 (83.3)	
Unknown	430	0	
T stage			
1	3258 (45.8)	4 (22.2)	0.044
2	2836 (39.8)	8 (44.4)	
3	540 (7.6)	3 (16.7)	
4	472 (6.6)	3 (16.7)	
X	15 (0.2)	0	
Unknown	11	0	
N stage			
0	4123 (58.0)	7 (38.9)	0.193
1	1730 (24.3)	8 (44.4)	
2	711 (10.0)	2 (11.1)	
3	506 (7.1)	1 (5.6)	
X	44 (0.6)	0	
Unknown	18	0	
M stage			
0	6834 (95.9)	16 (88.9)	0.166
1	290 (4.1)	2 (11.1)	
X	2 (0.03)	0	
Unknown	6	0	
Stage			
I	2548 (35.7)	2 (11.1)	0.054
II	2756 (38.6)	9 (50.0)	
III	1538 (21.6)	5 (27.8)	
IV	290 (4.1)	2 (11.1)	
ER status			
Negative	1867 (26.3)	12 (66.7)	<0.001
Positive	5219 (73.6)	6 (33.3)	
Equivocal	8 (0.1)	0	
Unknown	38	0	
PR status			
Negative	2676 (37.8)	12 (66.7)	0.015
Positive	4395 (62.1)	6 (33.3)	
Equivocal	6 (0.1)	0	
Unknown	55	0	
HER2 status			
Negative	4748 (68.3)	6 (33.3)	<0.001
Positive	1623(23.4)	12 (66.7)	
Equivocal	577 (8.3)	0	
Unknown	184	0	
Triple negative			
No	6180 (88.6)	14 (77.8)	0.141

	Controls, *n* (%)[a]	NF1-associated, *n* (%)[a]	*p*-value[b]
Yes	792 (11.4)	4 (22.2)	
Unknown	160	0	
Breast cancer subtype			
ER/PR positive, HER2 negative	3951 (62.1)	2 (11.1)	<0.001
HER2 positive	1623 (25.5)	12 (66.7)	
Triple negative	792 (12.4)	4 (22.2)	
Unknown	766	0	

[a] Unknown data were excluded from calculation of percentages.
[b] Unknown / X / Equivocal data were excluded from Fisher's exact test.

Table 3.3: Univariable analysis of overall survival.

Variable	No. of events / No. of patients	Median OS, years (95% CI)	Log-rank p-value	Hazard ratio (95% CI)	Cox model p-value
Overall	1073 / 6759	15.9 (12.1, UD)	NA	NA	NA
NF1 status					
Controls	1065 / 6741	15.9 (12.1, UD)	0.017	1	0.041
NF1	8 / 18	7.7 (4.7, UD)		2.29 (1.13, 4.61)	
Age at diagnosis, years	1073 / 6759	NA	NA	1.04 (1.03, 1.04)	<0.001
Tumour grade					
1	70 / 942	15.9 (UD)	<0.001	1	<0.001
2	296 / 2477	13.5 (12.1, UD)		1.83 (1.41, 2.38)	
3	569 / 2933	Not reached		3.16 (2.46, 4.06)	
T stage					
1	210 / 3027	15.9 (13.5, UD)	<0.001	1	<0.001
2	435 / 2721	Not reached		2.50 (2.12, 2.95)	
3	160 / 523	10.0 (9.7, UD)		5.37 (4.37, 6.59)	
4	249 / 462	4.6 (4.0, 5.1)		10.98 (9.13, 13.21)	
N stage					
0	331 / 3834	15.9 (13.5, UD)	<0.001	1	<0.001
1	326 / 1669	12.1 (11.1, UD)		2.34 (2.01, 2.73)	
2	172 / 698	10.4 (9.9, UD)		2.97 (2.47, 3.57)	
3	194 / 496	7.2 (6.5, 8.2)		5.20 (4.36, 6.22)	
M stage					
0	894 / 6468	15.9 (13.5, UD)	<0.001	1	<0.001
1	177 / 283	3.1 (2.9, 3.6)		7.98 (6.78, 9.39)	
Stage					
I	125 / 2332	15.9 (13.5, UD)	<0.001	1	<0.001
II	312 / 2640	Not reached		2.35 (1.91, 2.90)	
III	456 / 1504	10.0 (8.8, UD)		6.29 (5.16, 7.67)	
IV	180 / 283	3.0 (2.7, 3.4)		22.92 (18.21, 28.86)	
ER status					
Negative	425 / 1799	13.5 (10.6, UD)	<0.001	1	<0.001
Positive	636 / 4915	15.9 (12.1, UD)		0.53 (0.47, 0.60)	
PR status					
Negative	552 / 2565	12.1 (10.6, UD)	<0.001	1	<0.001
Positive	508 / 4137	Not reached		0.52 (0.46, 0.58)	
HER2 status					
Negative	660 / 4475	15.9 (12.1, UD)	0.002	1	0.003
Positive	277 / 1557	13.5 (11.1, UD)		1.25 (1.08, 1.43)	
Triple negative					
No	835 / 5838	15.9 (13.5, UD)	<0.001	1	<0.001
Yes	203 / 766	11.2 (10.6, UD)		2.16 (1.86, 2.52)	
Subtype					
ER/PR positive, HER2 negative	456 / 3704	15.9 (12.1, UD)	<0.001	1	<0.001
HER2 positive	277 / 1557	13.5 (11.1, UD)		1.53 (1.32, 1.78)	
Triple negative	203 / 766	11.2 (10.6, UD)		2.50 (2.12, 2.95)	

UD, undefined. Note: Non-proportional hazards were observed for tumour grade, T, N, M stage, overall stage, ER, PR status and breast cancer subtype.

Table 3.4: Multivariable analysis of overall survival.

Variable	Hazard ratio (95% CI)	p-value
NF1 status		
Controls	1	0.025
NF1	2.25 (1.11, 4.60)	
Age at diagnosis, years	1.03 (1.03, 1.04)	<0.001

Model was stratified by tumour grade (2 vs. 3), breast cancer stage (I / II / III vs. IV) and breast cancer subtype (ER / PR / HER2 positive vs. triple negative).
Grade 1 tumours were excluded from the model.

Mutation discovery

Next-generation sequencing (NGS) was performed on DNA extracted from peripheral blood with matched tumour DNA available for 16 patients (Figure 3.1, Supplementary Table 3.2). Ten out of 16 unique tumour samples passed quality control metrics with mean coverage of at least 50x, and were included in analysis of somatic mutations with the matched normal specimens. The pathogenicity of the variants were examined using *in silico* prediction tools, and variants predicted to be deleterious by at least one of the tools were identified (Supplementary Table 3.2).

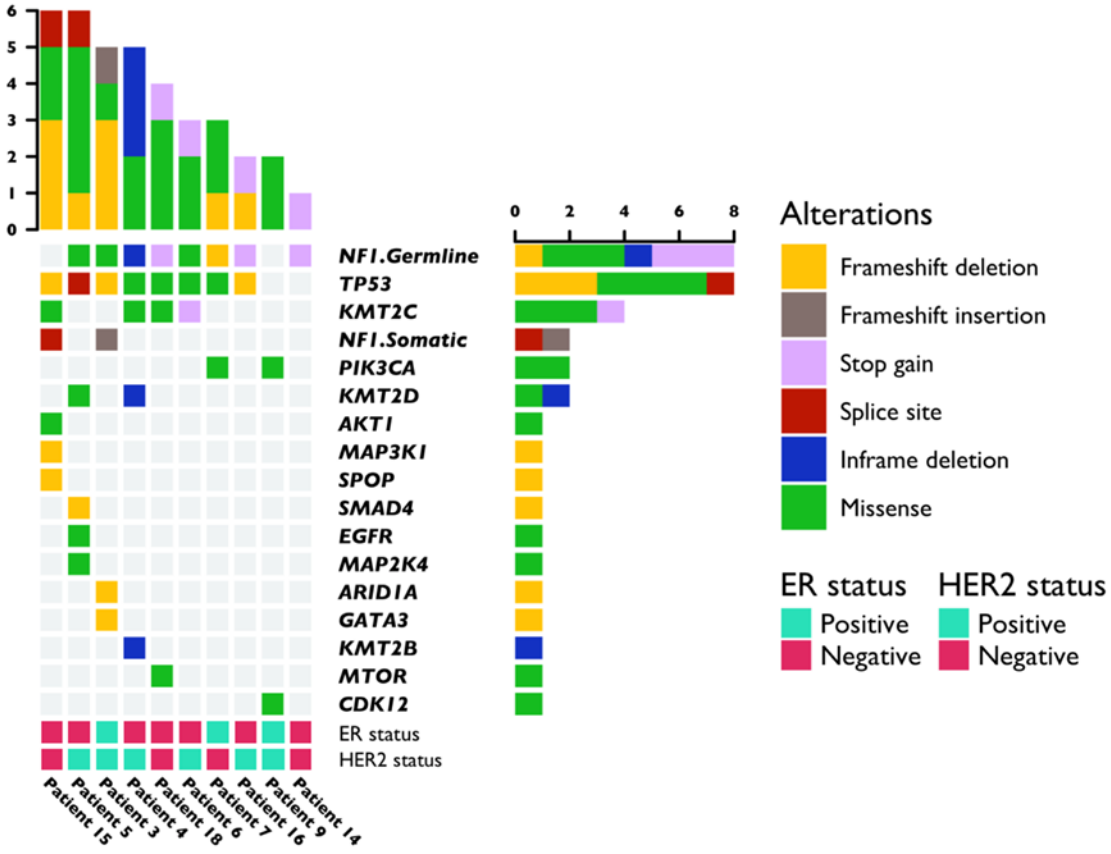


Figure 3.1: Oncoplot showing germline and somatic mutations in 10 NF1-associated breast cancers

We detected 10 different *NF1* germline mutations in 11 out of 16 (69%) patients. These comprised 3 missense variants (p.Y489C; p.L847P in 2 patients; p.Q1447H), 3 nonsense variants (p.R461*; p.Q2245*; p.Y2285*), 3 frameshift deletions (p.S2093fs;

p.T2284fs; p.Q2686fs) and one in-frame deletion variant (p.1446_1451del) (Table 3.1; Figure 3.1; Supplementary Table 3.2). We did not identify any complete/partial deletions or duplications of *NF1* with MLPA on the blood specimens negative for *NF1* mutation.

Mutations in *TP53* were observed in 8 out of 10 tumours (80%), all of which were pathogenic. We identified somatic "second-hit" *NF1* mutations (p.P678Pfs, c.3974+46_3974+47insG) in 2 of 10 patients (20%). In addition, somatic mutations were observed in *KMT2C* (40%), *KMT2D* (20%), and *PIK3CA* (20%) (Figure 3.1). Additional germline and somatic mutations were identified are listed in Supplementary Table 3.2.

The publicly available METABRIC breast cancer data [29,30] was reviewed to further explore the role of *NF1* and *TP53* alterations in sporadic breast cancers (Figure 3.2). Of the 2369 cases in the METABRIC dataset with sequence information, the frequency of *NF1* mutations was 4% (92/2369) overall, with no significant differences in frequency among the four different subtypes (hormone receptor (HR)-HER2+, triple negative, HR+HER2+ and HR+HER2- subtypes). However, when we included both *NF1* mutations and deletions (homozygous or heterozygous), the frequency of alterations in *NF1* was increased to 29% (590/2051) in unselected breast cancer cases, and was as high as 50% (146/292) in triple negative and 48% (62/130) in HR-HER2+ subtypes, followed by the HR+HER2+ subtype at 39% (41/105). Notably, the frequency of *NF1* alterations was significantly higher in HR-HER2+ and triple negative tumours compared to the HR+HER2- subtype at 21% (280/1344) (p<0.001) (Figure 3.2).

Remarkably, mutations of *TP53* frequently co-exist with *NF1* mutations in unselected sporadic breast cancers (p=0.041) (Figure 3.2). Co-existence of mutations in *NF1* and *KMT2C* was also observed (p=0.004). Moreover, the co-occurrence of alterations in *NF1* and *TP53* (p<0.001) as well as in *NF1* and *KMT2C* (p=0.006) remained statistically significant when both deletions and mutations were included.

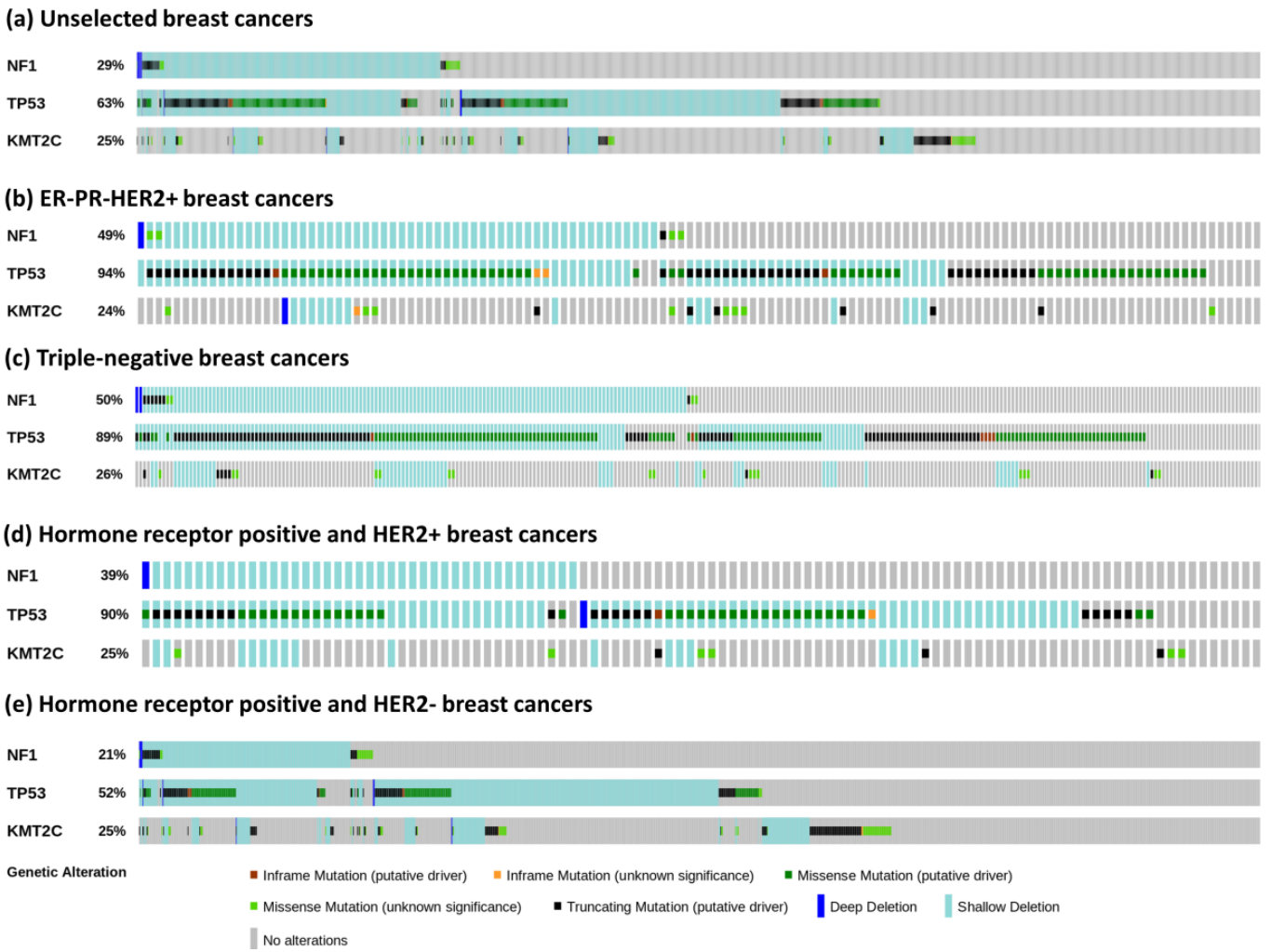


Figure 3.2: Frequency and co-existence of mutations and deletions in the *NF1*, *TP53* and *KMT2C* genes in (a) unselected breast cancers, (b) ER-negative, PR-negative, HER2-positive breast cancers , (c) triple negative breast cancers, (d) hormone receptor positive and HER2 positive breast cancers, (e) hormone receptor positive and HER2 negative breast cancers from the METABRIC database.

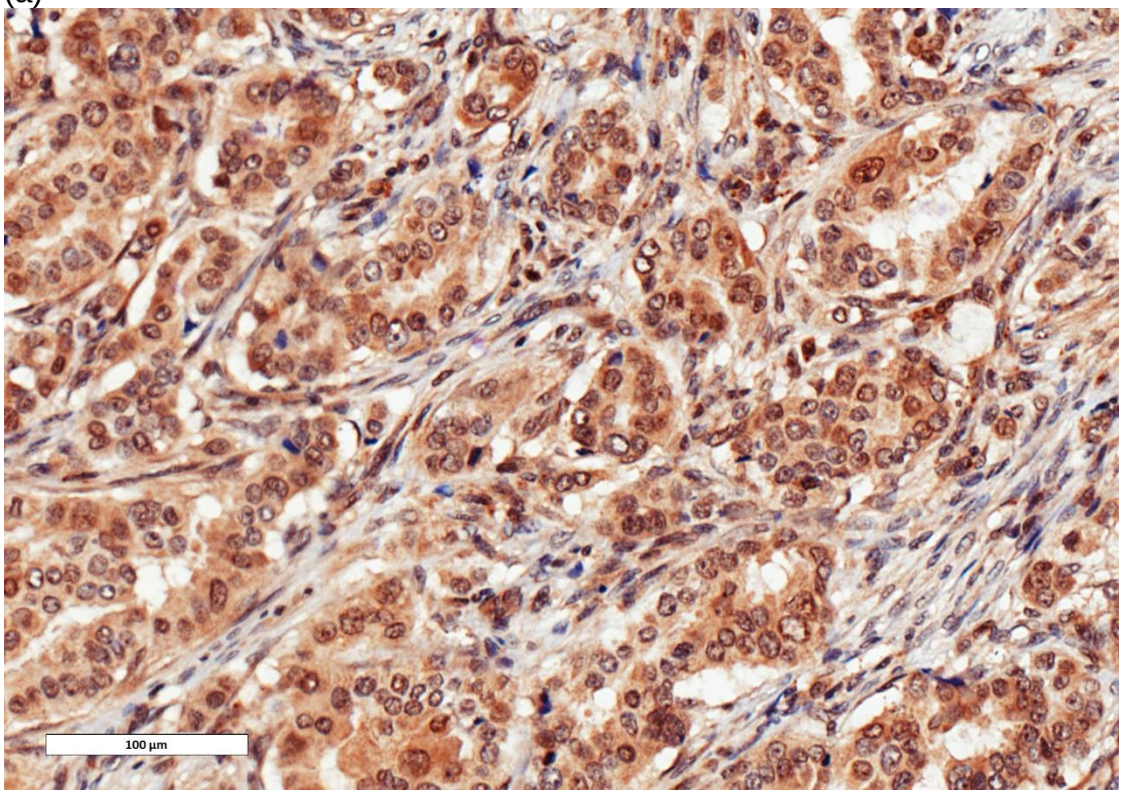
Immunohistochemical expression of neurofibromin

Immunohistochemical staining for neurofibromin was observed in the nuclei and/or cytoplasm of all 14 breast tumour specimens examined, although the percentage staining positive varied from 0% to 100% in either nuclei or cytoplasm (Table 3.5). We did not find complete loss of neurofibromin expression in any of the specimens, including 2 of the specimens which had somatic *NF1* mutations (Figure 3.3). In summary there was no obvious pattern in the immunohistochemical expression of neurofibromin based on *NF1* mutation or ER, PR, HER2 status.

Table 3.5: Immunohistochemical expression of neurofibromin

	Nuclear Staining (%)				Cytoplasmic Staining (%)				
Patient ID	**1+**	**2+**	**3+**	**Total%**	**1+**	**2+**	**3+**	**Total%**	**Subtype**
1	10	5	0	15	0	0	0	0	HER2
2	85	10	0	95	5	0	0	5	TNBC
3	5	0	0	5	20	0	0	20	HER2
5	10	5	0	15	20	5	0	25	HER2
7	60	15	0	75	10	0	0	10	Luminal
8	20	70	0	90	0	0	0	0	HER2
9	45	15	0	60	10	0	0	10	HER2
10	10	0	0	10	35	0	0	35	HER2
11	70	25	5	100	0	0	0	0	Luminal
12	0	0	0	0	5	0	0	5	HER2
13	5	0	0	5	5	0	0	5	HER2
15	70	20	0	90	10	0	0	10	TNBC
16	25	40	20	85	30	0	0	30	HER2
17	5	0	0	5	5	10	0	15	HER2

(a)



(b)

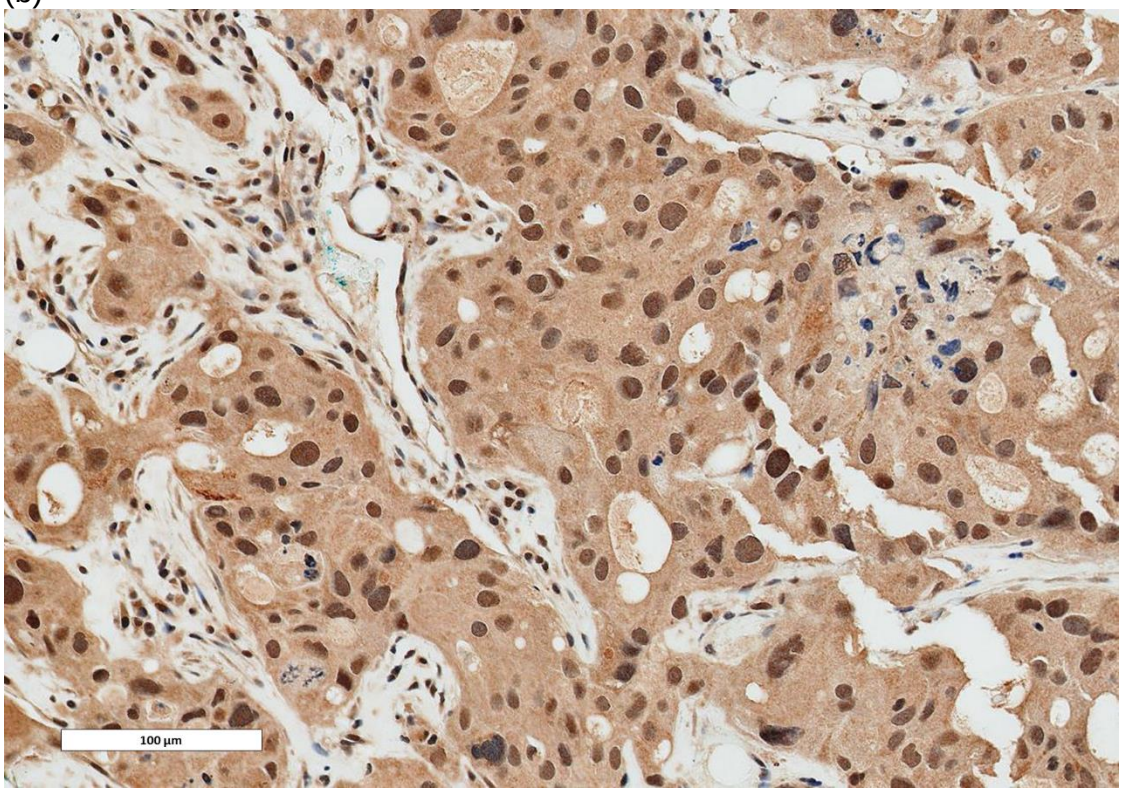


Figure 3.3: Immunohistochemical expression of neurofibromin in 2 breast cancer specimens with somatic second-hit *NF1* mutations: (a): Patient 3 (*NF1* mutations: germline p.L847P; somatic p.P678fs); (b): Patient 15 (*NF1* mutations: germline - not detected with DNA testing; somatic splicing c.3974+46_3974+47insG)

3.5 Discussion

The breast tumours in individuals with genetic predisposition disorders often have distinctive features, related to the underlying germline genetic defect (Table 3.6) [12,31-40]. In NF1, the breast cancers appear to be more aggressive than sporadic cancers, with a higher frequency of high grade tumours, hormone receptor negativity and HER2 overexpression (Table 3.6).

Table 3.6: Breast cancer characteristics (ER, PR, HER2) in breast cancer predisposition syndromes

Only the relative frequencies of the different subtypes from selected larger case series or studies are presented here, with the prominent features highlighted in bold.

Gene affected	Frequency of ER,PR, HER2 in invasive breast carcinomas	References
BRCA1	**Triple negative 61%**; ER+ 25%, PR+ 16%, HER2+ 7% **Triple negative 69%**; ER+ 22%, PR+ 21%, HER2+ 10%	31 32
BRCA2	Triple negative 10%; **ER+ 85%, PR+ 79%,** HER2+ 11% Triple negative 16%; **ER+ 77%, PR+ 64%,** HER2+ 13%	31 32
TP53	Triple negative 0%; ER+ 67%, PR+ 58%, **HER2+ 83%** ER-HER2- 5%; ER+ 84%, PR+ 72%, **HER2+ 63%** Triple negative 3%; ER/PR+HER2- 30%, **HER2+ 67%**	33 34 35
*CHEK2*1 100delC*	**ER+ 79%, PR+ 68%**, HER2+ 23% **ER+ 88%, PR+ 72%,** HER2+ 21% **ER+ 87%, PR+ 73%,** HER2+ 16%	36 37 38
PALB2 1592delT	**Triple negative 55%**; ER and/or PR+HER2- 41%, HER2+ 5% **Triple negative 30%**; ER+ 74%	39 40
NF1	Triple negative 22%; **ER- 67%, PR- 67%, HER2+ 67%** Triple negative 19%; **ER- 54%, PR- 65%, HER2+ 31%**	This study 12

This is the first study to incorporate the clinical, pathological and genomic characteristics of NF1-associated breast cancer in a comprehensive manner. To date, there has been little data in the literature on breast cancer in NF1 patients. However, a recent publication based on data from the Finnish Cancer and NF Registry [12]

supports our earlier finding [14]. Similar to our findings, it reported a higher frequency of unfavourable prognostic features among 26 breast cancer cases, including HER2 amplification, hormone receptor negativity, higher grade and larger tumour size, compared to matched controls in the general population (Table 3.6) [12]. Combining the grade, ER, PR and HER2 information from our 18 cases with the 26 cases reported by Uusitalo et al [12], out of total of 44 NF1-associated breast cancers, 66% (29/44) are grade 3, 59% (26/44) are ER negative, 66% (29/44) are PR negative, and 45% (20/44) are HER2 positive. Similar to our study, survival outcomes were also worse than the sporadic cancers [12]. Although the number of patients in our comprehensive case series is limited, all patients who relapsed or died from breast cancer had HER2 positive disease. Moreover, HER2-directed therapies and cytotoxic therapies appear to have limited efficacy in these patients from our experience (Table 3.1). Both patients with de novo stage 4 HER2 positive breast cancer died within 6 months of diagnosis in spite of taxane and trastuzumab combination treatment; two out of three stage 3 HER2 positive tumours relapsed while on maintenance trastuzumab. These findings strongly suggest that haploinsufficiency of *NF1*, a critical tumour suppressor gene, contributes to treatment resistance and suboptimal tumour control with standard therapies, resulting in inferior cancer survival outcomes.

To elucidate any other therapeutic targets for treating NF1-associated breast cancers, next generation sequencing was performed on matched blood and tumour specimens. In addition to detection of the underlying germline *NF1* mutations, germline variants in other cancer-related genes were also detected in our series of patients. A study which focused on germline genomic profiling of 14 women with NF1 and breast cancer by Wang *et al* did not find deleterious mutations in other high/moderate-penetrance breast cancer genes (*ATM, BRCA1, BRCA2, BARD1, BRIP1, CDH1, CHEK2, FANCC, MRE11A, NBN, PALB2, PTEN, RAD50, RAD51C, TP53,* and *STK11*), but discovered 25 rare or common variants in other cancer related genes [41]. It is possible that these variants may contribute to increasing the risk of breast cancer in women with NF1.

A striking finding from the somatic profiling in our study is the detection of deleterious *TP53* mutations in 80% of the tumours. This is consistent with the hypothesis that cooperation between *NF1* and *TP53* can trigger carcinogenesis, and result in the development of aggressive biology. The co-existence of *NF1* and *TP53* mutations has

also been observed in other sporadic tumours such as glioblastomas, melanomas and ovarian carcinomas [3,42-45], in addition to the sporadic breast cancers from the METABRIC dataset (Figure 3.2).

Apart from *TP53* mutations, *NF1* mutations may cooperate with mutations in other genes such as *KMT2C*, in the pathogenesis of these NF1-associated breast tumours. Mutations or alterations in *NF1* also frequently co-exist with *KMT2C* mutations or alterations in breast cancers from the METABRIC database (Figure 3.2). *KMT2C* (Lysine Methyltransferase 2C), also known as *MLL3*, is a member of the histone lysine methyltransferase family. It is frequently altered in various cancers, and increasing evidence supports its role as a tumour suppressor [46]. *KMT2C* encodes a DNA-binding protein that methylates histone H3 lys4 (H3K4), leading to an open chromatin structure with activation of target gene expression [46]. In acute myeloid leukaemia, a recent study demonstrated that haploinsufficiency in *MLL3*, together with *NF1* suppression and *TP53* deficiency, promoted leukaemogenesis in mouse and human systems. Interestingly, these leukaemias were refractory to conventional chemotherapy, but sensitive to JQ1, a BET (bromodomain and extra-terminal) inhibitor [47]. A number of studies have also reported that somatic mutations, losses or reduced expression of *KMT2C* were associated with inferior survival outcomes in breast cancer [48-50].

Review of the publicly available METABRIC breast cancer data [29,30](Figure 3.2) showed that *NF1* aberrations are more frequent in HR negative HER2 positive and triple negative tumours compared to HR positive HER2 negative tumours. Importantly, the presence of inactivating mutations in *NF1* was associated with inferior breast cancer-specific survival among ER negative tumours in this METABRIC dataset (hazard ratio 2.7; CI: 1.3-5.5) [30]. A recent study on the genomic evolution of breast cancer metastases also reported that *NF1* mutations were significantly enriched in the metastatic specimens [51], in keeping with the important role that *NF1* plays in breast cancer pathogenesis and progression.

One of the limitations of our study is the inability to evaluate copy number aberrations, since fresh tissue specimens were not available for the majority of patients. The detection of mutations in *NF1* can also be challenging, even with next generation sequencing. Apart from its large size with 60 exons spanning over 350kb, the *NF1* gene has one of the highest mutation rates with a myriad of possible mutations, and

the presence of several pseudogenes can further complicate the detection of *NF1* mutations. We detected germline *NF1* mutations in 69% of the clinically diagnosed NF1 patients, and this success rate is typical of studies with DNA-based methods alone detecting less than 80% of germline mutations [2]. The addition of MLPA testing may only detect approximately 4% of patients with deletion or duplication of single or multiple exons [19,52]. Splicing mutations may be present in more than 20% of individuals with NF1 syndrome, hence analysis of RNA is essential to detect these intronic mutations [52,53]. A protein-based approach may help to detect deficiency of neurofibromin. However, immunohistochemical expression of neurofibromin in these NF1-associated breast tumours did not reveal any distinctive pattern of staining. None of the tumours examined had total absence of neurofibromin, in contrast to a study using the same antibody, which reported complete absence in 15-18% of sporadic melanomas [54]. Tumoural heterogeneity may account for the variability in neurofibromin expression, but current antibodies available are also unlikely able to distinguish between the normal and mutant neurofibromin protein, especially in the case of missense mutations.

3.6 Conclusions

Our study confirms the aggressive nature of breast cancers in patients with *NF1*, with a high frequency of grade 3, ER negative and HER2 positive tumours. The mutation profile of the tumours strongly suggest that germline *NF1* mutations, in cooperation with *TP53* and other genes such as *KMT2C*, play a critical role in the pathogenesis of these tumours. This study also highlights the poor response of these tumours to standard cytotoxic and HER2 therapeutic approaches; hence more effective therapeutic strategies need to be developed.

3.7 References

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3.8 Supplementary data

Supplementary Table S3.1: Gene panels

SureSelect custom gene panel (338 genes) (Gene symbol)

ABCC11	CARD11	EGFR	FGF4	INPP4B	MSH2	PHLPP2	SDHD	TRIM17
ABL1	CASP5	EML4	FGF6	IRF4	MSH6	PIK3CA	SERPINI2	TSC1
AKT1	CASP8	EP300	FGFR1	IRS2	MTOR	PIK3CG	SETD2	TSC2
AKT2	CBFB	EPCAM	FGFR2	JAK1	MUTYH	PIK3R1	SF3B1	TSHR
AKT3	CBL	EPHA3	FGFR3	JAK2	MYC	PIK3R2	SLAMF6	VHL
ALK	CCND1	EPHA5	FGFR4	JAK3	MYCL1	PMS2	SLC26A10	VPS13B
APC	CCND2	EPHB1	FH	JUN	MYCN	POLD1	SMAD2	WISP3
AR	CCND3	EPS8L1	FHIT	KDM5A	MYD88	POLE	SMAD3	WRN
ARAF	CCNE1	ERBB2	FILIP1	KDM5C	MYH9	PPEF2	SMAD4	WT1
ARFRP1	CD79A	ERBB3	FLT1	KDM6A	MYST3	PPP2R1A	SMARCA4	XPO1
ARID1A	CD79B	ERBB4	FLT3	KDR	NBN	PRDM1	SMARCB1	XRCC2
ARID1B	CDC73	ERG	FLT4	KEAP1	NCOR1	PRKAR1A	SMARCD1	ZFP36L1
ARID2	CDH1	ESR1	FOXA1	KIAA1919	NF1	PRKDC	SMARCD2	ZIM2
ASXL1	CDH5	ETV1	FOXL2	KIT	NF2	PRMT7	SMO	ZNF217
ATM	CDK12	ETV4	FTMT	KLHL6	NFE2L2	PRSS1	SOCS1	ZNF451
ATR	CDK4	ETV5	GATA1	KRAS	NFKBIA	PRSS7	SOSTDC1	ZNF582
ATRX	CDK6	ETV6	GATA2	LIG4	NKX2-1	PTCH1	SOX10	ZNF668
AURKA	CDK8	EWSR1	GATA3	LRP1B	NOD2	PTCHD3	SOX2	ZNF703
AURKB	CDKN1A	EZH2	GID4	MAGI3	NOTCH1	PTEN	SPEN	
AXIN1	CDKN1B	FAM123B	GNA11	MAP2K1	NOTCH2	PTPN11	SPINK1	
AXL	CDKN2A	FAM46C	GNA13	MAP2K2	NOTCH3	PTPN22	SPOP	
BAP1	CDKN2B	FAM47C	GNAQ	MAP2K4	NOTCH4	PTPRF	SRC	
BARD1	CDKN2C	FAN1	GNAS	MAP3K1	NPM1	RAD50	SSX9	
BCL2	CEBPA	FANCA	GPR124	MAP3K13	NRAS	RAD51C	STAG2	
BCL2L1	CFHR5	FANCB	GPS2	MAPKAP1	NTRK1	RAD51D	STAT3	
BCL2L2	CHEK1	FANCC	GRIN2A	MCAT	NTRK2	RAF1	STAT4	
BCL6	CHEK2	FANCD2	GSK3B	MCL1	NTRK3	RARA	STK11	
BCOR	CIC	FANCE	HEATR7B2	MDM2	NUP93	RB1	SUFU	
BCORL1	CREBBP	FANCF	HGF	MDM4	OR2L2	RET	TAOK1	
BCR	CRKL	FANCG	HIF1A	MED12	OR6A2	RICTOR	TBX3	
BLM	CRLF2	FANCI	HRAS	MEF2B	PAK1	RINT1	TET2	
BMPR1A	CSF1R	FANCL	HSP90AA1	MEN1	PAK3	RNF43	TGFBR2	
BRAF	CTCF	FANCM	IDH1	MET	PALB2	ROS1	TLR4	
BRCA1	CTNNA1	FBXW7	IDH2	MITF	PALLD	RPTOR	TMPRSS2	
BRCA2	CTNNB1	FETUB	IGF1R	MLH1	PAX5	RUNX1	TNFAIP3	
BRIP1	CXCL6	FGF10	IGSF22	MLL	PBRM1	RUNX1T1	TNFAIP6	
BRIX1	DAXX	FGF14	IKBKE	MLL2	PDGFRA	SDHA	TNFRSF14	
BTK	DDR2	FGF19	IKZF1	MLL3	PDGFRB	SDHAF2	TNK2	
C11ORF30	DNMT3A	FGF23	IL7R	MPL	PDK1	SDHB	TOP1	
C2ORF63	DOT1L	FGF3	INHBA	MRE11A	PDPK1	SDHC	TP53	

xGen® Pan-Cancer Panel (127 genes) (Gene Symbol)

ACVR1B	EPPK1	NPM1	TSHZ2
ACVR2A	ERBB4	NRAS	TSHZ3
AJUBA	ERCC2	NSD1	U2AF1
AKT1	EZH2	PBRM1	USP9X
APC	FBXW7	PCBP1	VEZF1
AR	FGFR2	PDGFRA	VHL
ARHGAP35	FGFR3	PHF6	WT1
ARID1A	FLT3	PIK3CA	
ARID5B	FOXA1	PIK3CG	
ASXL1	FOXA2	PIK3R1	
ATM	GATA3	POLQ	
ATR	H3F3C	PPP2R1A	
ATRX	HGF	PRX	
AXIN2	HIST1H1C	PTEN	
B4GALT3	HIST1H2BD	PTPN11	
BAP1	IDH1	RAD21	
BRAF	IDH2	RB1	
BRCA1	KDM5C	RPL22	
BRCA2	KDM6A	RPL5	
CBFB	KEAP1	RUNX1	
CCND1	KIT	SETBP1	
CDH1	KRAS	SETD2	
CDK12	LIFR	SF3B1	
CDKN1A	LRRK2	SIN3A	
CDKN1B	MALAT1	SMAD2	
CDKN2A	MAP2K4	SMAD4	
CDKN2C	MAP3K1	SMC1A	
CEBPA	MAPK8IP1	SMC3	
CHEK2	MECOM	SOX17	
CRIPAK	MIR142	SOX9	
CTCF	KMT2B	SPOP	
CTNNB1	KMT2C	STAG2	
DNMT3A	KMT2D	STK11	
EGFR	MTOR	TAF1	
EGR3	NAV3	TBL1XR1	
EIF4A2	NCOR1	TBX3	
ELF3	NF1	TET2	
EP300	NFE2L2	TGFBR2	
EPHA3	NFE2L3	TLR4	
EPHB6	NOTCH1	TP53	

Supplementary Table S3.2: List of mutations detected from blood and tumour in NF1 patients

Chapter 4
Immunohistochemical expression of neurofibromin in sporadic breast cancers

4.1 Abstract

NF1 (Neurofibromatosis type 1) is one of the significantly mutated genes in many cancers, including breast cancer. However, the clinical relevance of neurofibromin deficiency in sporadic breast cancer is unclear. We hypothesised that loss of expression neurofibromin, a tumour suppressor, will be associated with overexpression of pAkt and pMAPK downstream in the PI3K-MAPK pathway, resulting in worse outcomes. The expression of neurofibromin was initially evaluated through immunohistochemistry on microarrayed cores obtained from 314 stage 1-3 breast cancer specimens diagnosed between 2000 and 2002. “Positive” expression of neurofibromin was defined as nuclear and cytoplasmic staining in 10% or more of tumour cells. Positive expression of neurofibromin, as defined above, was seen in 44.6% (140/314) of tumours. Staining for neurofibromin was observed in a median of 5% (0-95%) of cells for nuclear staining, and 40% (0-95%) of cells for cytoplasmic staining. “Negative” expression of neurofibromin was associated with high tumour grade ($p<0.001$), hormone receptor negativity ($p<0.001$), lymph node positivity ($p=0.041$) and larger tumour size ($p=0.031$). “Negative” expression of neurofibromin was also associated with increased risk of relapse (5-year relapse rate 29.2% vs 17.0%; hazard ratio 1.56, $p=0.029$) and death (5-year death rate 21.7% vs 12.7%; hazard ratio 1.64, $p=0.029$) on univariate analysis. On multivariate analysis, lack of neurofibromin was an independent predictor of relapse and death for triple negative cancers (hazard ratios 3.33, $p=0.011$ and 2.94, $p=0.026$ respectively), but not in the luminal and HER2 positive immunohistochemical subtypes. To validate this finding, the expression of neurofibromin was subsequently evaluated in tissue microarrays with a total of 594 triple negative breast cancers diagnosed from 1993 to 2011. No association between neurofibromin expression and survival outcomes was found in the validation set. Immunohistochemistry may be suboptimal for assessing deficiency or dysfunction of neurofibromin. Novel methods such as mass-spectrometry-based proteomic analyses may potentially be superior for this purpose.

4.2 Introduction

NF1 (Neurofibromatosis type 1) is one of the significantly mutated genes in many cancers, including breast cancer [1-3]. However, given the large size of the gene with lack of mutational hotspots, there are challenges with detecting *NF1* mutations and interpreting the functional significance of the variants detected [1]. Loss of *NF1*, especially heterozygous loss, is more common, and may potentially lead to activation of the downstream PI3K-Akt-MAP kinase pathway, since its product neurofibromin is an important negative regulator of Ras cellular proliferation pathways[1,4]. Loss of *NF1* was implicated as a critical breast cancer driver in a study looking at the mammary tumours generated in Chaos3 mice models, which exhibit high levels of genomic instability [5]. The role of *NF1* in breast cancer pathogenesis and progression is increasingly recognized. A recent study has also reported inferior survival outcomes in hormone-receptor positive tumours harbouring *NF1* nonsense or frameshift mutations [6].

Given the potential role of *NF1* and neurofibromin in breast cancer, as well as the limitations in evaluation of its deficiency from DNA-based studies, we sought to investigate the immunohistochemical expression of neurofibromin in sporadic breast cancers. In addition, there may be downregulation of the neurofibromin tumour suppressor protein via epigenetic mechanisms[1], hence the immunohistochemical staining of neurofibromin may serve as a useful surrogate of the downstream protein expression. To our knowledge, the immunohistochemical expression of neurofibromin has only been evaluated in a study on tissue microarrays (TMAs) containing 22 sporadic breast cancers, 18 benign lesions and 6 normal breast tissue specimens. There were no differences in expression of neurofibromin in the various tissues[7].

We hypothesise that loss of expression of neurofibromin, a tumour suppressor, will be associated with overexpression of pAkt and pMAPK downstream in the PI3K-MAPK pathway, resulting in worse outcomes. The objectives of the study were to evaluate the immunohistochemical expression of neurofibromin in breast cancer, and its association with clinocopathological features and survival outcomes. We also aimed to evaluate the association of neurofibromin expression with immunohistochemical expression of pAkt and pMAPK.

4.3 Methods

This study used TMAs constructed from 347 sporadic breast cancers diagnosed between April 2000 and Dec 2002 at the Department of Anatomical Pathology, Singapore General Hospital for the discovery set, and from 681 triple negative breast cancers diagnosed over the period 1993 to 2011 for the validation set. The tumours used for the discovery and validation sets were independent, that is, no overlap. Using a Beecher microarrayer with 1mm punch, at least two representative areas of the tumour in each FFPE tissue block were constructed into the TMAs. This study was approved by SingHealth Centralised Institutional Review Board.

The TMA sections (4µm) were subjected to immunohistochemical staining with antibodies to neurofibromin (ab30325, 1:300 dilution; Abcam, Cambridge, UK), phospho-Akt (pAkt) (#3787, clone 736E11, 1:50 dilution; Cell Signaling Technology Danvers, MA, USA) and pMAPK (Phospho-Thr202/Tyr204, #4370, 1:400 dilution; Cell Signaling Technology, Danvers, MA, USA), according to manufacturers' instructions. These experiments were undertaken in the pathology department at the Singapore General Hospital, a diagnostic laboratory which also conducts academic research, including immunohistochemical studies using various antibodies. These antibodies were chosen based on various references in the literature[7-10], including the study by Maertens et al which implicated loss of NF1 with activation of Akt and ERK signaling in melanomas as a mechanism of resistance to BRAF inhibitors[8]. Tonsillar tissue, which is known to express low neurofibromin protein within the germinal centres (https://www.proteinatlas.org/ENSG00000196712-NF1/tissue/tonsil), was used as positive control. The immunohistochemical assessment was performed independently by two observers (including the candidate) blinded to the clinicopathological characteristics and survival outcomes. For immunoscoring, the staining intensity (nil, weak (1+), moderate (2+), strong (3+)) and the proportion of tumour cells stained in the nuclei as well as the cytoplasm were recorded separately.

"Positive" expression of neurofibromin was defined as nuclear and cytoplasmic staining in 10% or more of tumour cells, considering that tumour suppressor proteins including neurofibromin may shuttle between the nucleus and the cytoplasm for

regulation of various cellular functions [11]. Tumours which did not fulfil this criterion were classified as “negative” for expression of neurofibromin. Expression of neurofibromin, pAkt and pMAPK in nuclei and cytoplasm was scored separately.

Details on clinicopathologic status, including tumour grade, size, nodal and lymphovascular invasion status as well as survival outcomes (relapse status and overall survival status) were obtained from medical records. Tumours that were stage 4 at diagnosis were excluded from the analyses. The definitions of estrogen receptor (ER) and progesterone receptor (PR) in this study were based on the latest recommendations by the American Society of Clinical Oncology and the College of American Pathologists [12], but human epidermal growth factor receptor 2 (HER2) positivity definitions were based on the earlier definitions by the American Society of Clinical Oncology and the College of American Pathologists in 2007 [13].

Continuous variables were summarised using median and range, while categorical variables were summarised by the number and the percentage of patients in each category. Relapse-free survival (RFS) was calculated as the time from diagnosis to disease relapse or death from any cause. Overall survival (OS) was defined as time from diagnosis to death from any cause. OS, rather than breast cancer-specific survival (BCSS), was used as an endpoint of survival outcome in this analysis. OS is often regarded as one of the most robust endpoints and frequently adopted in epidemiological studies and clinical trials. Unlike OS, which can be easily and accurately determined from medical records or death registry, BCSS may be subject to bias and inaccuracies as cause of death may not be attributed correctly to the contributing cause. Patients who were alive and relapse-free at last follow-up were censored at date of last follow-up. The Kaplan-Meier method was used to estimate the failure function, from which 5-year relapse rates were derived. The log-rank test was used to determine if there was a difference in RFS and OS between different groups of patients. The Cox proportional hazards model was used to estimate the hazard ratios between groups. A 2-sided *p*-value of less than 0.05 was taken as statistically significant. All analyses were performed in Stata (Version 12.1, StataCorp, Texas, USA).

4.4 Results

Discovery Set

Out of the 347 stage 1-3 sporadic breast cancers used in the TMAs, 175 (50.4%) were "luminal" (defined as hormone receptor positive and HER2 negative), 74 (21.3%) were HER2-positive (regardless of hormone receptor status), and 95 (27.4%) were triple negative. Other clinicopathological characteristics are displayed in Table 4.1. A total of 314 stage 1-3 breast cancer specimens in this series had adequate tissue for evaluating the expression of neurofibromin through immunohistochemistry (Table 4.1). Median follow-up was 132.2 months.

"Positive" expression of neurofibromin, as defined above, was seen in 44.6% (140/314) of tumours. "Negative" expression of neurofibromin was associated with high tumour grade ($p<0.001$), hormone receptor negativity ($p<0.001$), lymph node positivity ($p=0.041$) and larger tumour size ($p=0.031$) (Table 4.1). Figure 4.1 shows 3 different specimens with varying expression of neurofibromin (mild, moderate, strong).

Staining for neurofibromin was observed in a median of 5% (0-95%) of cells for nuclear staining, and 40% (0-95%) of cells for cytoplasmic staining (Table 4.2). The percentage of cells staining positive for pMAPK and pAkt, as well as the H scores, are shown in Table 4.2. Examples of specimens with varying immunohistochemical expression of pMAPK and pAkt are shown in Figure 4.2.

Table 4.1: Clinicopathological parameters by neurofibromin (NF1) expression (Positive expression of neurofibromin was defined as both nuclear and cytoplasmic staining in 10% or more of tumour cells; tumours which did not fulfil this criterion were classified as "negative" for expression of neurofibromin.)

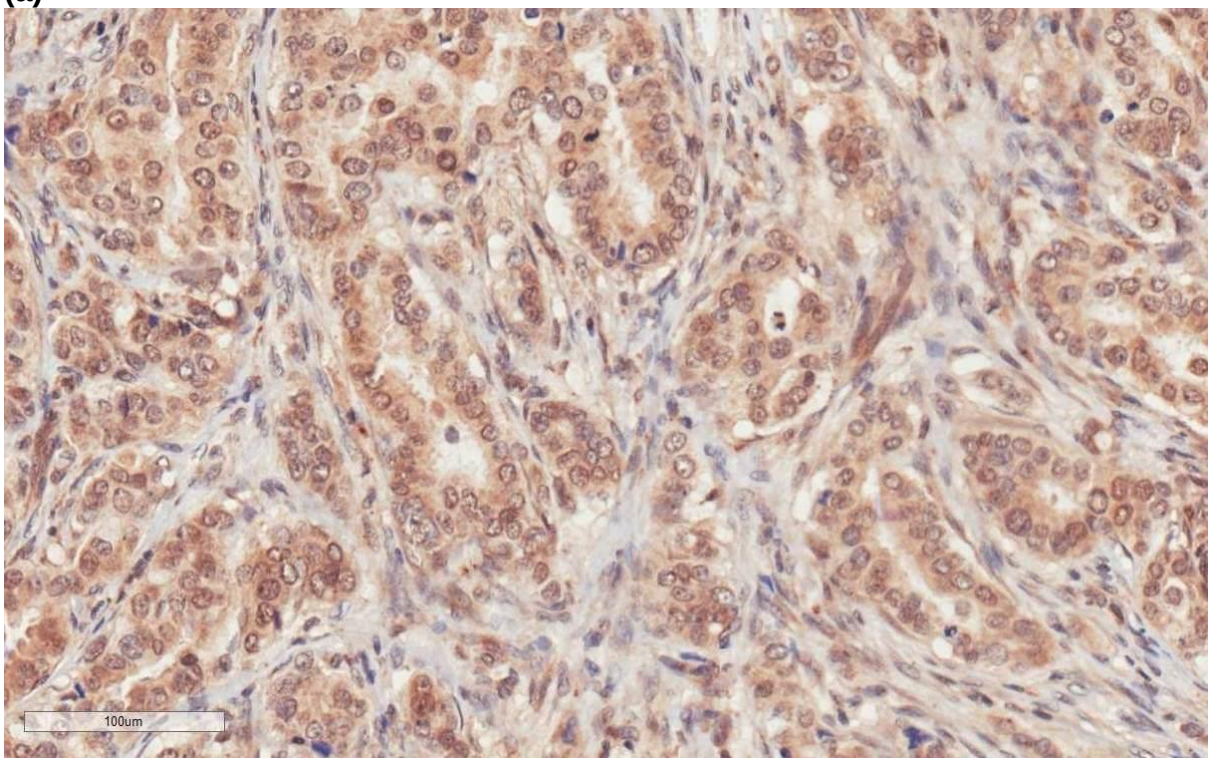
	Total (N=347)		NF1 negative (N=174)		NF1 positive (N=140)		
Parameter	**Number**	**%**	**Number**	**%**	**Number**	**%**	**p-value**
Age at diagnosis, years							0.191
Mean	53.3		54.2		52.4		
Median	51		53		50.5		
Range	23 – 85		29 – 85		23 – 84		
Race							0.004
Chinese	291	83.9	136	78.2	125	89.3	
Malay	31	8.9	26	14.9	5	3.6	
Indian	16	4.6	9	5.2	6	4.3	
Others	9	2.6	3	1.7	4	2.9	
Tumour size, mm							0.031
Mean	31.1		34.1		28.4		
Median	25		29		23		
Range	2 – 200		3 – 200		2 – 200		
Tumour grade							< 0.001
1	64	18.4	21	12.1	34	24.3	
2	130	37.5	55	31.6	64	45.7	
3	145	41.8	96	55.2	38	27.1	
Unknown	8	2.3	2	1.2	4	2.9	
Histologic subtype							ND
Invasive ductal carcinoma (IDC)	312	89.9	160	92.0	123	87.9	
Invasive lobular carcinoma (ILC)	12	3.5	5	2.9	7	5.0	
IDC and ILC	1	0.3	1	0.6	0	0	
Invasive cribriform carcinoma	2	0.6	0	0	2	1.4	
Medullary carcinoma	2	0.6	2	1.1	0	0	
Metaplastic carcinoma	1	0.3	1	0.6	0	0	
Mucinous carcinoma	9	2.6	2	1.1	4	2.9	
Papillary carcinoma	4	1.2	2	1.1	2	1.4	
Tubular carcinoma	4	1.2	1	0.6	2	1.4	
Lymphovascular invasion							0.579
Absent	278	80.1	136	78.2	113	80.7	
Present	69	19.9	38	21.8	27	19.3	
Nodal stage							0.041
0	175	50.4	78	44.8	78	55.7	
1	86	24.8	45	25.9	35	25.0	
2	43	12.4	29	16.7	10	7.1	
3	33	9.5	19	10.9	11	7.9	

Parameter	Total (N=347)		NF1 negative (N=174)		NF1 positive (N=140)		p-value
	Number	%	Number	%	Number	%	
Unknown	10	2.9	3	1.7	6	4.3	
ER status							< 0.001
Negative	147	42.4	92	52.9	45	32.1	
Positive	197	56.8	82	47.1	92	65.7	
Unknown	3	0.9	0	0	3	2.1	
PR status							< 0.001
Negative	173	49.9	107	61.5	51	36.4	
Positive	171	49.3	67	38.5	86	61.4	
Unknown	3	0.9	0	0	3	2.1	
HER2 status							0.329
Negative	270	77.8	133	76.4	111	79.3	
Positive	74	21.3	41	23.6	26	18.6	
Unknown	3	0.9	0	0	3	2.1	
Molecular subtype							0.001
Luminal	175	50.4	70	40.2	84	60.0	
HER2 positive	74	21.3	41	23.6	26	18.6	
Triple negative	95	27.4	63	36.2	27	19.3	
Unknown	3	0.9	0	0	3	2.1	

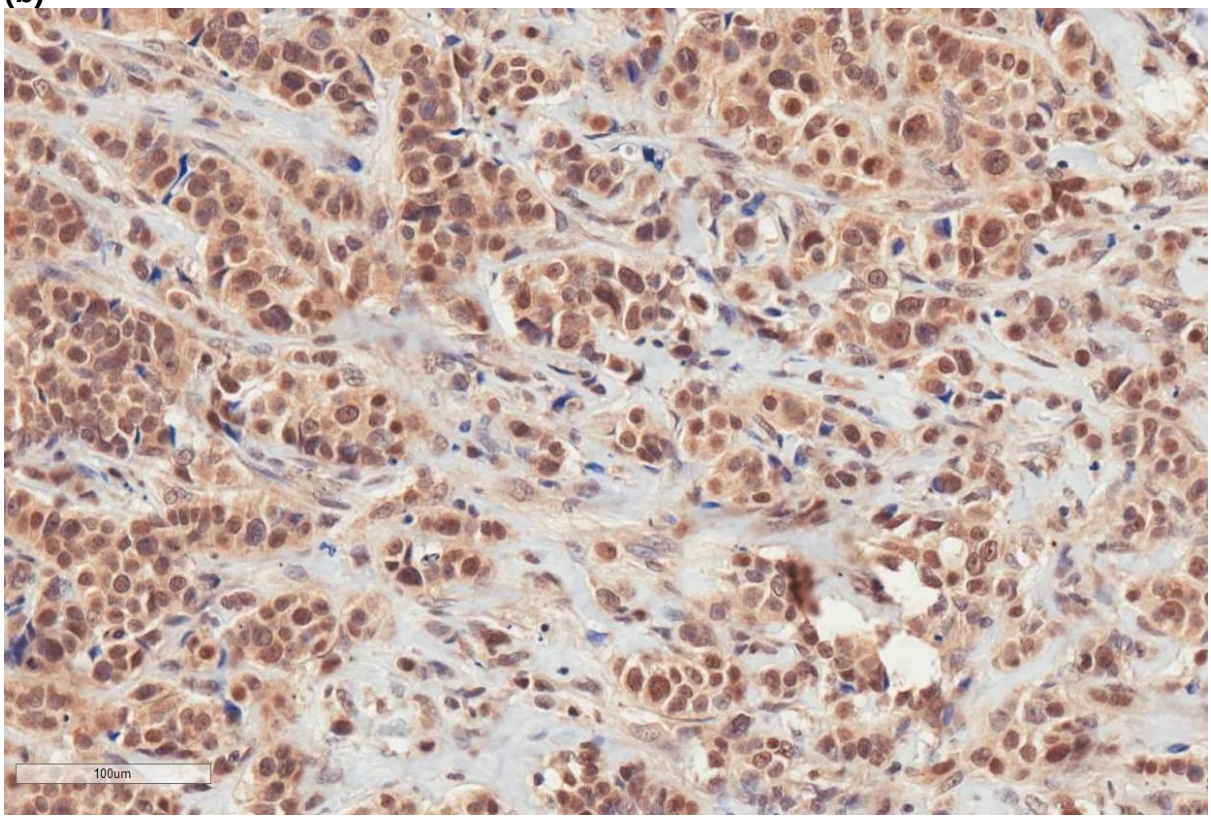
Note: 33 patients (9.5%) had unknown neurofibromin staining status due to lack of adequate tissue.
ND: Not done.

Figure 4.1: Sporadic breast cancer specimens demonstrating (a) mild, (b) moderate and (c) strong expression of neurofibromin

(a)



(b)



(c)

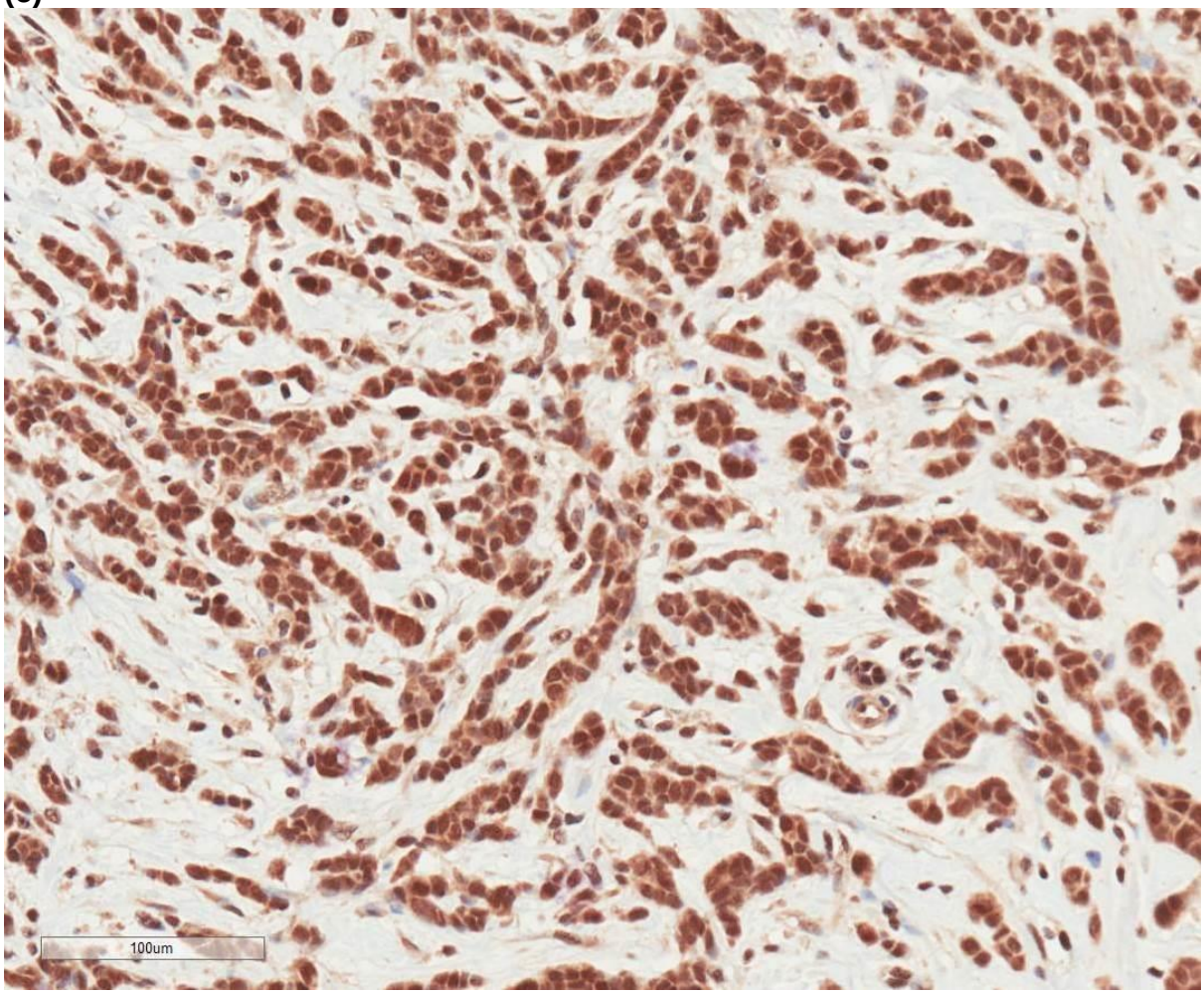
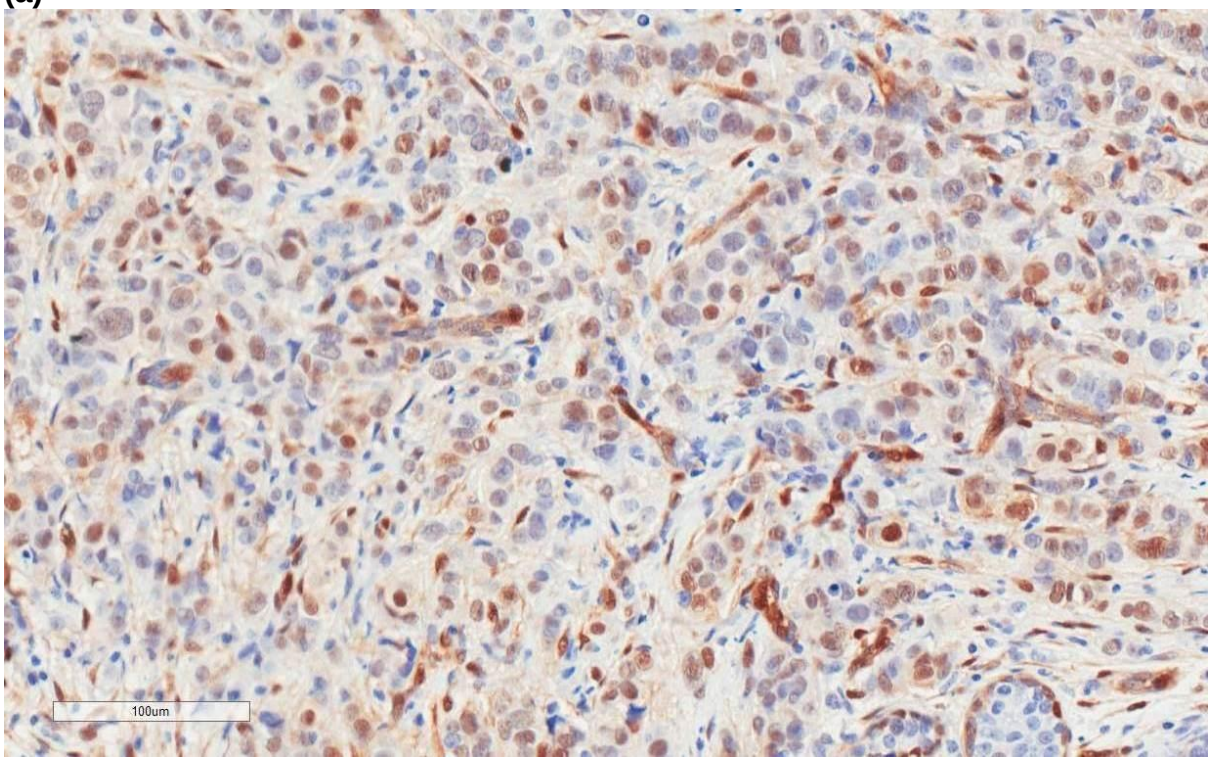


Table 4.2: Immunostaining of neurofibromin (NF1), pMAPK and pAKt in nuclei and cytoplasm for discovery set.

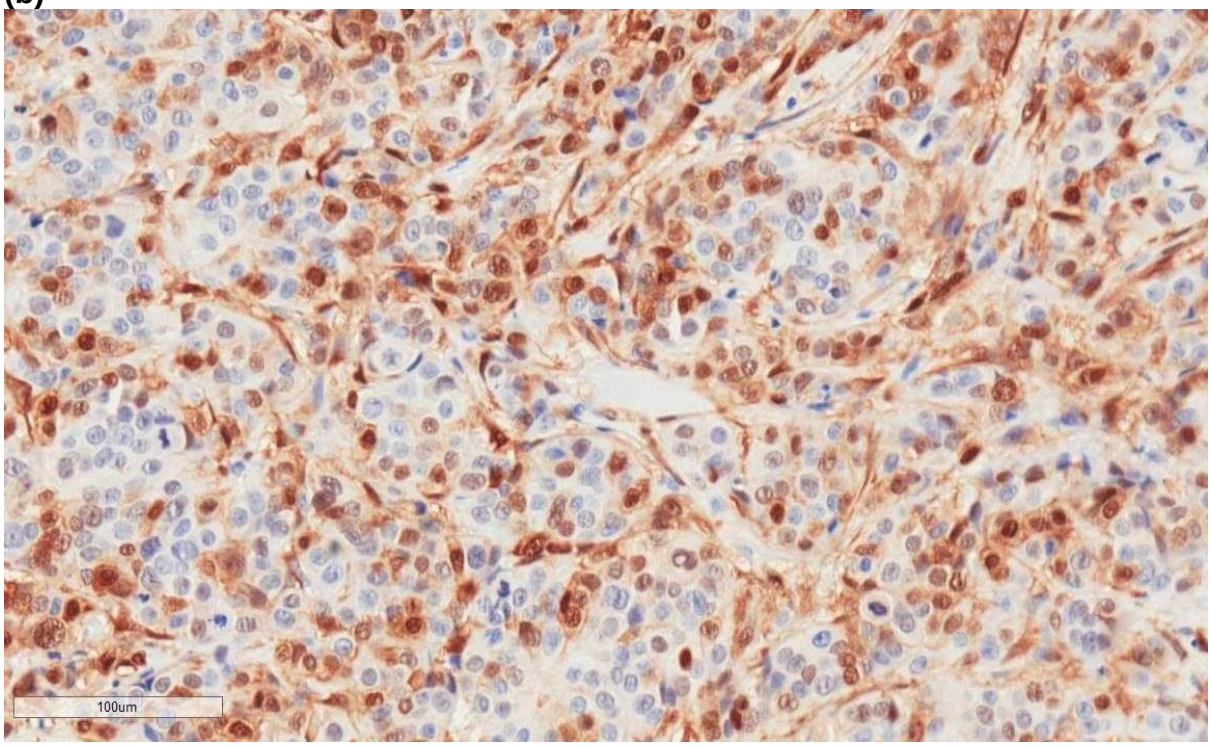
	Median percentage staining positive (range)	**%**
NF1 (nucleus)	5 (0 – 95)	
Unknown	33	9.3
NF1 (cytoplasm)	40 (0 – 95)	
Unknown	33	9.3
pMAPK (nucleus)	5 (0 – 90)	
Unknown	18	5.1
pMAPK (cytoplasm)	0 (0 – 80)	
Unknown	18	5.1
pAKT (nucleus)	0 (0 – 80)	
Unknown	13	3.7
pAKT (cytoplasm)	5 (0 – 70)	
Unknown	13	3.7
	H-score: Median (range)	**%**
NF1 (nucleus)	5 (0 – 175)	
Unknown	33	9.3
NF1 (cytoplasm)	40 (0 – 160)	
Unknown	33	9.3
pMAPK (nucleus)	5 (0 – 230)	
Unknown	18	5.1
pMAPK (cytoplasm)	0 (0 – 155)	
Unknown	18	5.1
pAKT (nucleus)	0 (0 – 80)	
Unknown	13	3.7
pAKT (cytoplasm)	5 (0 – 85)	
Unknown	13	3.7

Figure 4.2: Sporadic breast cancer specimens demonstrating (a) mild, (b) moderate and (c) strong expression of pMAPK, and (d) mild, (e) moderate and (f) strong expression of pAkt.

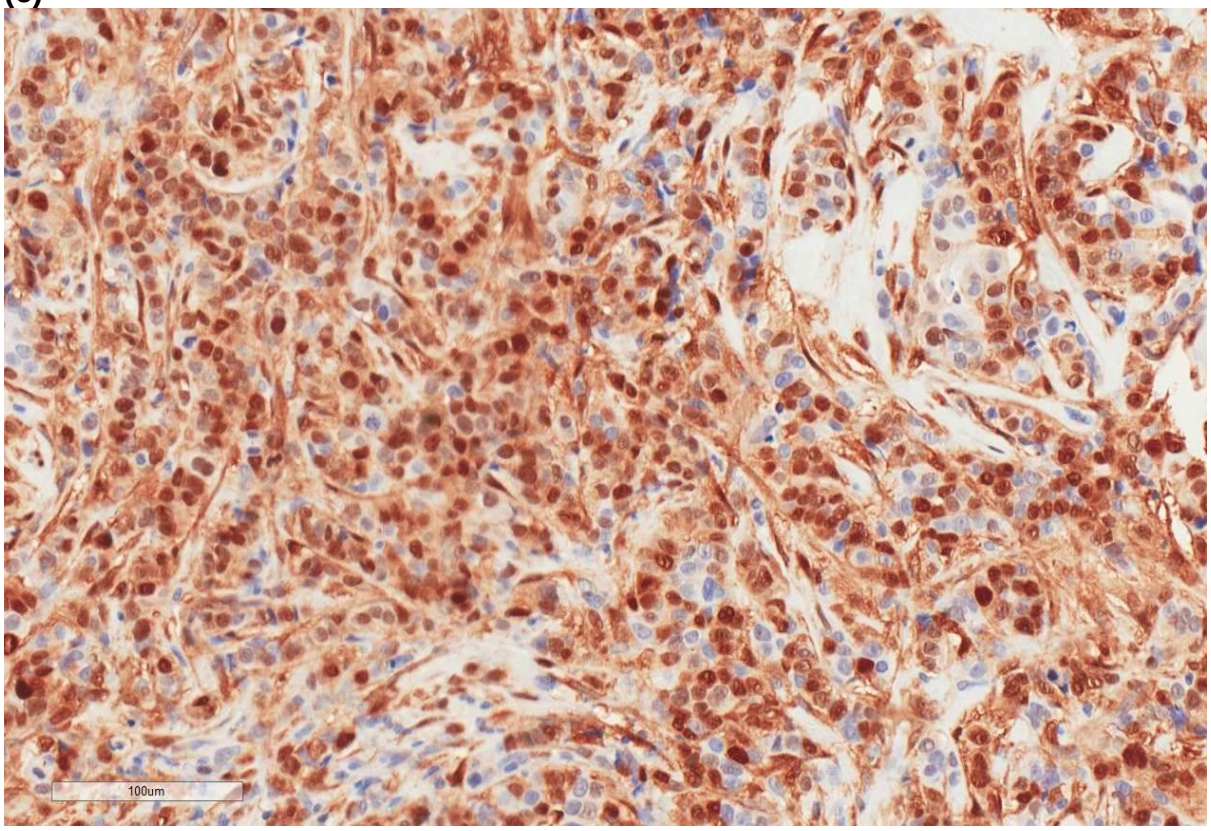
(a)



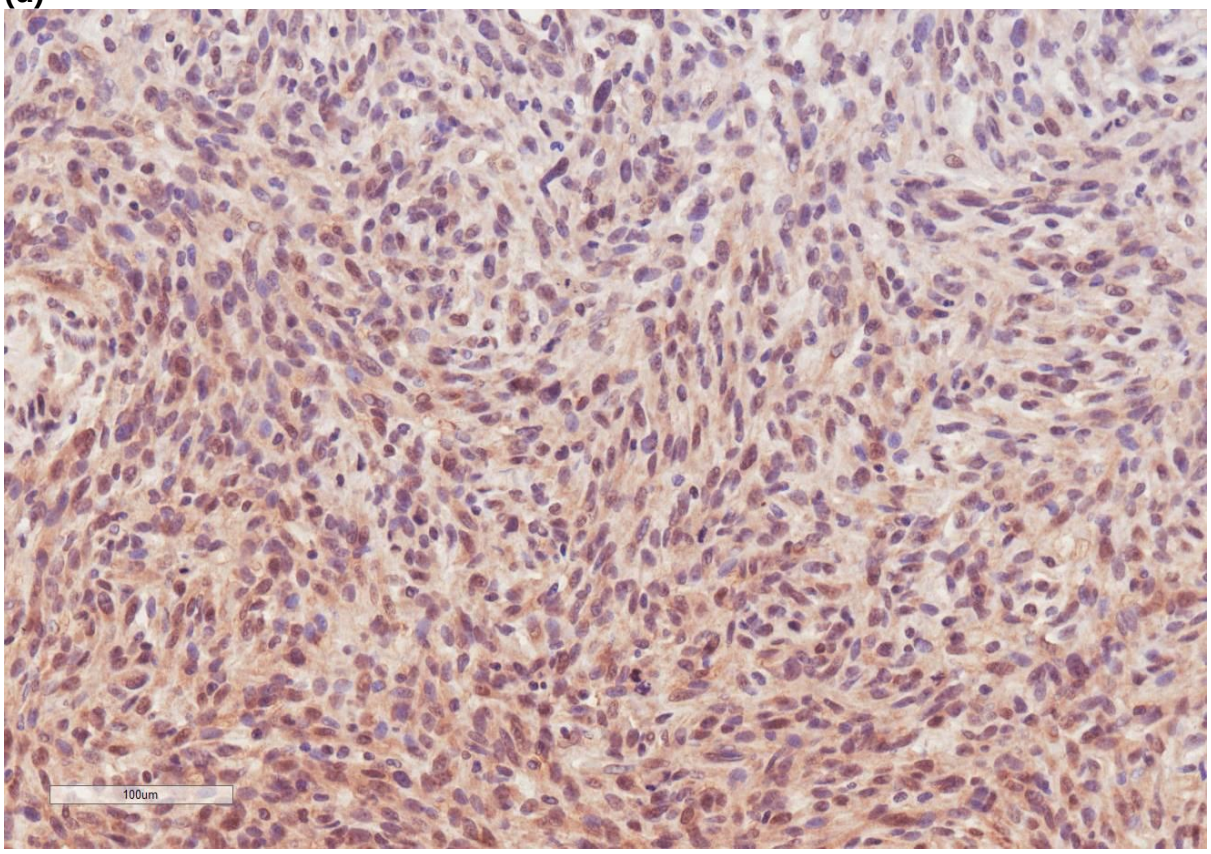
(b)



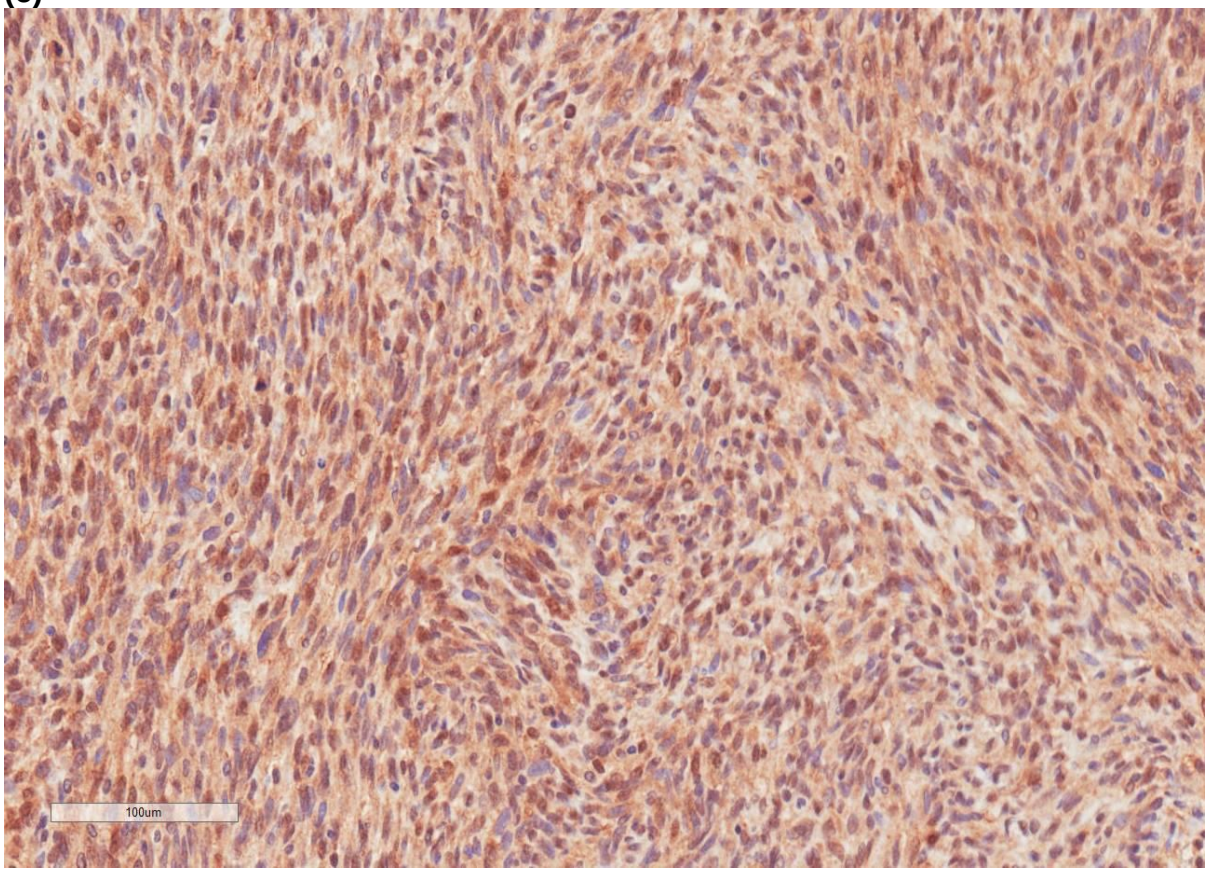
(c)



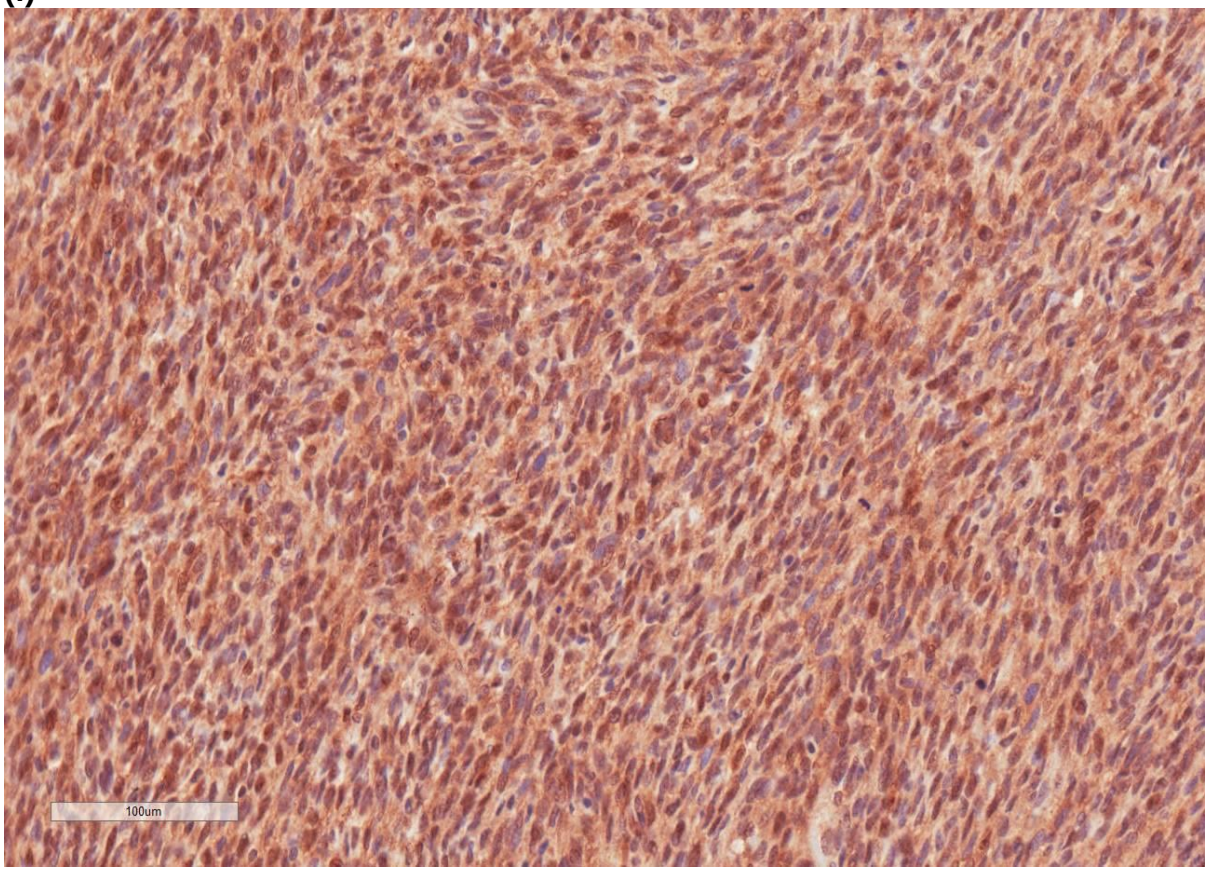
(d)



(e)



(f)



“Negative” expression of neurofibromin was also associated with increased risk of relapse (5-year relapse rate 29.2% vs 17.0%; hazard ratio 1.56, p=0.029) (Table 4.3) and death (5-year death rate 21.7% vs 12.7%; hazard ratio 1.64, p=0.029) on univariate analysis (Table 4.4). As demonstrated in the relapse-free survival and overall survival curves (Figures 4.3 and 4.4), the prognostic role of neurofibromin seemed most prominent in the triple negative subtype.

On multivariate analysis, lack of neurofibromin was an independent predictor of relapse and death for triple negative cancers (hazard ratios 3.33, p=0.011 and 2.94, p=0.026 respectively), but not in the luminal and HER2 positive immunohistochemical subtypes (Table 4.5 and Table 4.6).

In general, there was no evidence that loss of NF1 was associated with increased pAKT or pMAPK expression (Table 4.7 and Table 4.8). On the contrary, there was evidence that NF1, pAKT and pMAPK expressions were positively correlated; Spearman’s correlations were all positive. The strongest association found was between nuclear NF1 and nuclear pAKT (Table 4.7).

Table 4.3: Univariate analysis of relapse-free survival

	No. of events / patients	5-year relapse rate, % (95% CI)	p-value[1]	Hazard ratio (95% CI)	p-value[2]
All patients	114 / 346	23.8 (19.6 – 28.7)	NA	NA	NA
Tumour size, cm	114 / 346	NA	NA	1.13 (1.08 – 1.18)	< 0.001
Tumour grade			0.001		< 0.001
1	10 / 63	7.9 (3.4 – 18.0)		1	
2	44 / 130	20.1 (14.0 – 28.3)		2.63 (1.32 – 5.24)	
3	58 / 145	34.1 (26.9 – 42.6)		3.43 (1.75 – 6.72)	
Nodal stage			< 0.001		< 0.001
0	37 / 175	13.5 (9.2 – 19.7)		1	
1	29 / 85	23.7 (16.0 – 34.3)		1.76 (1.08 – 2.87)	
2	20 / 43	34.3 (21.9 – 51.1)		2.83 (1.63 – 4.89)	
3	23 / 33	60.3 (43.8 – 77.2)		6.11 (3.61 – 10.35)	
Lymphovascular invasion			0.001		0.002
Absent	81 / 277	20.9 (16.5 – 26.3)		1	
Present	33 / 69	35.3 (25.2 – 47.9)		1.97 (1.31 – 2.95)	
Molecular subtype			0.044		0.046
Luminal	50 / 174	17.0 (12.2 – 23.6)		1	
HER2 positive	28 / 74	31.0 (21.6 – 43.2)		1.52 (0.95 – 2.41)	
Triple negative	36 / 95	31.5 (23.0 – 42.1)		1.65 (1.07 – 2.54)	
NF1 expression			0.030		0.029
Negative	64 / 174	29.2 (22.9 – 36.7)		1	
Positive	38 / 139	17.0 (11.6 – 24.4)		0.64 (0.43 – 0.96)	
Luminal					
NF1 expression			0.346		0.348
Negative	21 / 70	20.8 (12.9 – 32.6)		1	
Positive	21 / 83	12.2 (6.8 – 21.5)		0.75 (0.41 – 1.37)	
HER2 positive					
NF1 expression			0.615		0.617
Negative	15 / 41	35.8 (23.0 – 52.8)		1	
Positive	12 / 26	32.2 (17.6 – 54.3)		1.22 (0.57 – 2.61)	

	No. of events / patients	5-year relapse rate, % (95% CI)	p-value[1]	Hazard ratio (95% CI)	p-value[2]
Triple negative					
NF1 expression			0.031		0.021
Negative	28 / 63	34.4 (23.9 – 47.7)		1	
Positive	5 / 27	19.4 (8.5 – 40.6)		0.36 (0.14 – 0.95)	

[1] Log-rank test

[2] Likelihood ratio test (from Cox model)

NA: Not applicable.

Table 4.4: Univariate analysis of overall survival

	No. of events / patients	5-year survival rate, % (95% CI)	p-value[1]	Hazard ratio (95% CI)	p-value[2]
All patients	93 / 346	17.4 (13.8 – 22.0)	NA	NA	NA
Tumour size, cm	93 / 346	NA	NA	1.15 (1.09 – 1.20)	< 0.001
Tumour grade			0.004		0.002
1	8 / 63	6.4 (2.4 – 16.0)		1	
2	36 / 130	17.1 (11.5 – 25.1)		2.68 (1.24 – 5.78)	
3	47 / 145	23.1 (16.9 – 31.1)		3.35 (1.58 – 7.10)	
Nodal stage			< 0.001		< 0.001
0	29 / 175	10.7 (6.9 – 16.5)		1	
1	22 / 85	14.4 (8.4 – 24.0)		1.68 (0.97 – 2.93)	
2	17 / 43	19.7 (10.3 – 35.6)		2.93 (1.60 – 5.36)	
3	20 / 33	51.0 (35.0 – 69.2)		6.12 (3.44 – 10.89)	
Lymphovascular invasion			0.007		0.011
Absent	66 / 277	15.1 (11.3 – 20.0)		1	
Present	27 / 69	26.9 (17.8 – 39.2)		1.84 (1.18 – 2.88)	
Molecular subtype			0.030		0.031
Luminal	39 / 174	11.2 (7.3 – 17.0)		1	
HER2 positive	23 / 74	21.8 (13.7 – 33.5)		1.59 (0.95 – 2.66)	
Triple negative	31 / 95	26.5 (18.6 – 36.9)		1.82 (1.14 – 2.93)	
NF1 expression			0.031		0.029
Negative	54 / 174	21.7 (16.2 – 28.8)		1	
Positive	31 / 139	12.7 (8.1 – 19.7)		0.61 (0.39 – 0.96)	
Luminal					
NF1 expression			0.306		0.309
Negative	17 / 70	13.3 (7.1 – 23.9)		1	
Positive	16 / 83	8.6 (4.2 – 17.2)		0.70 (0.35 – 1.39)	
HER2 positive					
NF1 expression			0.686		0.687
Negative	13 / 41	21.3 (11.2 – 38.1)		1	
Positive	10 / 26	28.9 (14.9 – 51.3)		1.19 (0.52 – 2.72)	
Triple negative					
NF1 expression			0.074		0.059
Negative	24 / 63	31.4 (21.3 – 44.8)		1	

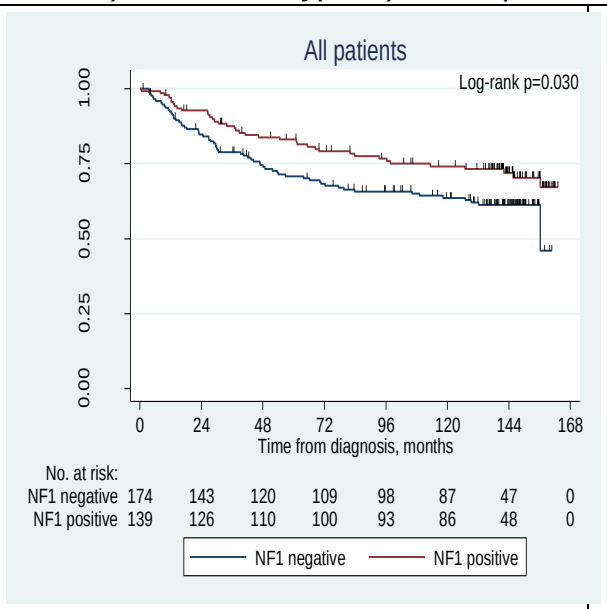
	No. of events / patients	5-year survival rate, % (95% CI)	p-value[1]	Hazard ratio (95% CI)	p-value[2]
Positive	5 / 27	11.8 (3.9 – 32.3)		0.43 (0.16 – 1.12)	

[1] Log-rank test

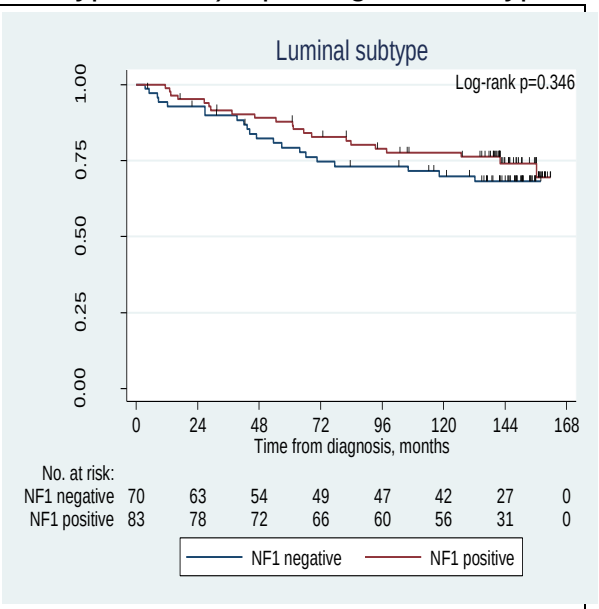
[2] Likelihood ratio test (from Cox model)

NA: Not applicable.

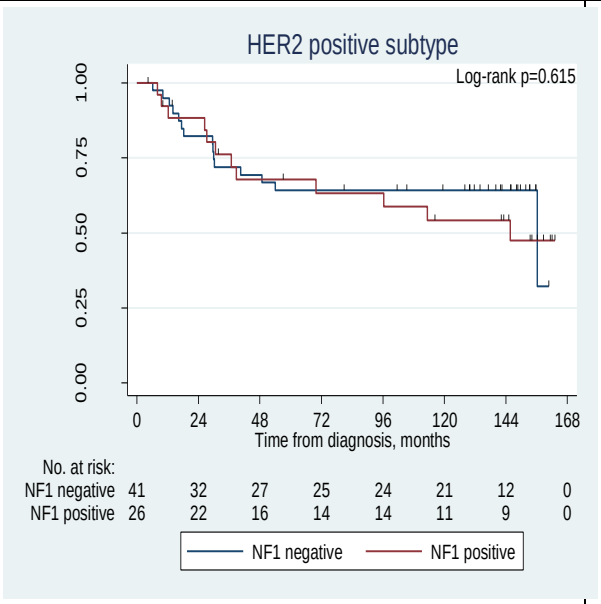
Figure 4.3: Relapse free survival according expression of neurofibromin in a) overall cohort, b) luminal subtype, c) HER2-positive subtype and d) triple negative subtype.



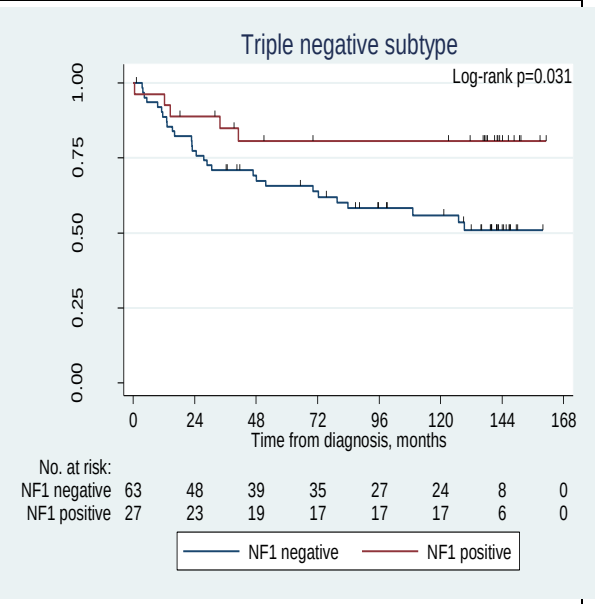
a) *Kaplan-Meier plot of relapse-free survival by neurofibromin expression in all patients*



b) Kaplan-Meier plot of relapse-free survival by neurofibromin expression in luminal patients

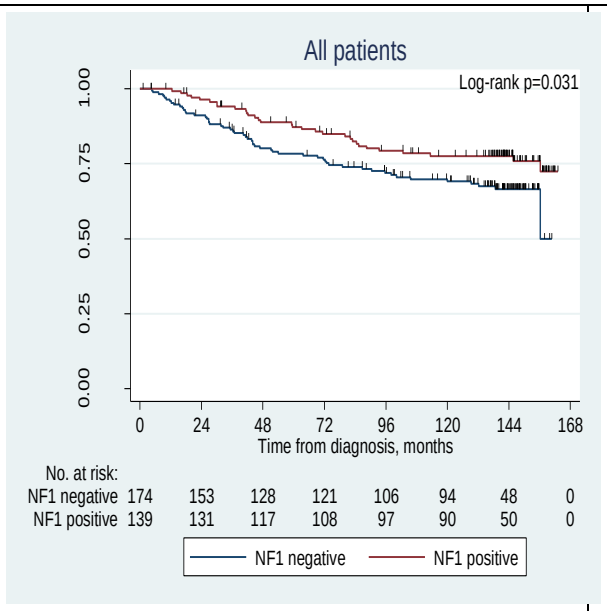


c) Kaplan-Meier plot of relapse-free survival by neurofibromin expression in HER2 positive patients

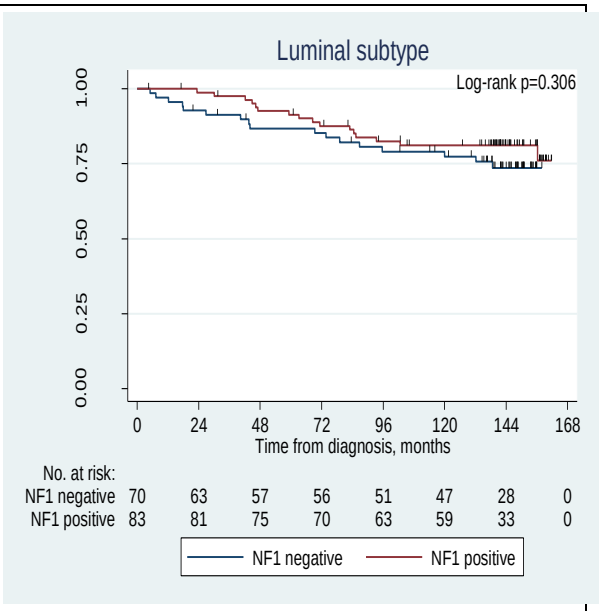


d) Kaplan-Meier plot of relapse-free survival by neurofibromin expression in triple negative patients

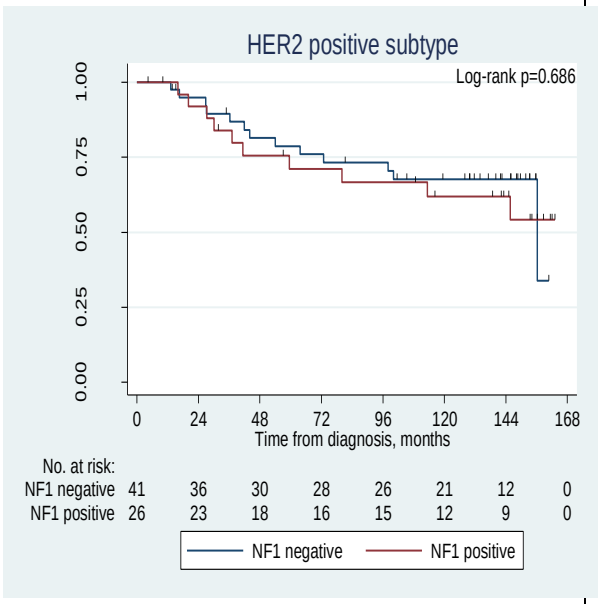
Figure 4.4: Overall survival according expression of neurofibromin in a) overall cohort, b) luminal subtype, c) HER2-positive subtype and d) triple negative subtype.



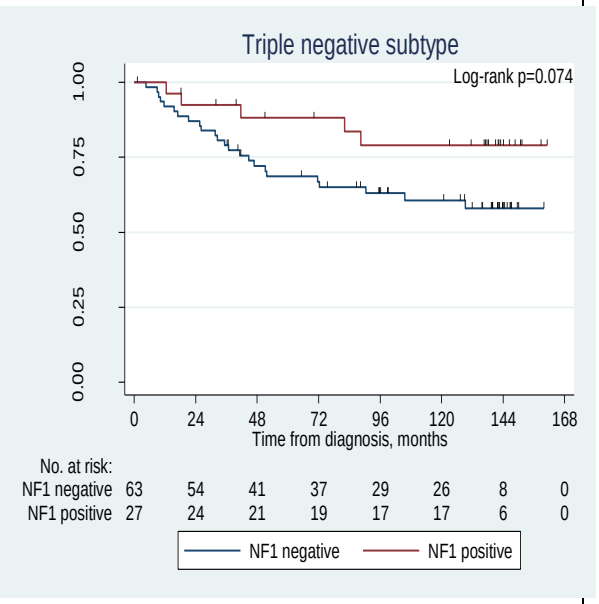
a) Kaplan-Meier plot of overall survival by NF1 expression in all patients



b) Kaplan-Meier plot of overall survival by NF1 expression in luminal patients



c) Kaplan-Meier plot of overall survival by NF1 expression in HER2 positive patients



d) Kaplan-Meier plot of overall survival by NF1 expression in triple negative patients

Table 4.5: Multivariate models of RFS by breast cancer subtype

	Luminal (Events = 39, Patients = 146)		HER2 positive (Events = 25, Patients = 63)		Triple negative (Events = 32, Patients = 87)	
	Hazard ratio (95% CI)	***p*[1]**	**Hazard ratio (95% CI)**	***p*[1]**	**Hazard ratio (95% CI)**	***p*[1]**
Tumour size, cm	1.22 (1.05 – 1.42)	0.015	1.46 (1.15 – 1.84)	0.004	1.03 (0.94 – 1.13)	0.560
Tumour grade		0.104		0.773	Not included[2]	
1	1		1			
2 – 3	2.17 (0.80 – 5.89)		1.33 (0.18 – 10.06)			
Nodal stage		0.003		0.230		0.033
0 – 1	1		1		1	
2 – 3	3.16 (1.52 – 6.58)		1.95 (0.69 – 5.53)		2.27 (1.10 – 4.66)	
Lymphovascular invasion		0.879		0.331		0.014
Absent	1		1		1	
Present	1.06 (0.51 – 2.22)		0.57 (0.17 – 1.87)		2.56 (1.25 – 5.24)	
NF1 expression		0.705		0.867		0.011
Negative	1		1		1	
Positive	1.14 (0.57 – 2.28)		1.07 (0.48 – 2.41)		0.30 (0.11 – 0.87)	

[1] p-value from likelihood ratio test

[2] Hazard ratio for tumour grade was inestimable as there were no events amongst the Grade 1 patients in the triple negative subset. Therefore, it was excluded from the model.

Table 4.6: Multivariate models of OS by breast cancer subtype

	Luminal (Events = 30, Patients = 146)		HER2 positive (Events = 21, Patients = 63)		Triple negative (Events = 28, Patients = 87)	
	Hazard ratio (95% CI)	***p*[1]**	**Hazard ratio (95% CI)**	***p*[1]**	**Hazard ratio (95% CI)**	***p*[1]**
Tumour size, cm	1.32 (1.11 – 1.56)	0.002	1.52 (1.18 – 1.96)	0.004	1.03 (0.92 – 1.14)	0.643
Tumour grade		0.327		0.991	Not included[2]	
1	1		1			
2 – 3	1.73 (0.55 – 5.38)		0.99 (0.13 – 7.61)			
Nodal stage		0.009		0.073		0.118
0 – 1	1		1		1	
2 – 3	3.17 (1.35 – 7.41)		2.89 (0.96 – 8.66)		1.90 (0.88 – 4.12)	
Lymphovascular invasion		0.838		0.355		0.085
Absent	1		1		1	
Present	0.92 (0.39 – 2.15)		0.57 (0.16 – 1.97)		2.05 (0.94 – 4.49)	
NF1 expression		0.947		0.834		0.026
Negative	1		1		1	
Positive	0.97 (0.45 – 2.11)		1.10 (0.45 – 2.69)		0.34 (0.12 – 0.99)	

[1] p-value from likelihood ratio test

[2] Hazard ratio for tumour grade was inestimable as there were no events amongst the Grade 1 patients in the triple negative subset. Therefore, it was excluded from the model.

Table 4.7: Association between pAKT, pMAPK and nuclear NF1 expression (using a cut-off of 10% of cells staining positive)

Parameter	Total (N=347) Number	%	Nuclear NF1 negative (N=170) Number	%	Nuclear NF1 positive (N=144) Number	%	ρ	p-value
Cytoplasmic NF1							0.21	< 0.001
Negative	30	8.7	26	15.3	4	2.8		
Positive	284	81.8	144	84.7	140	97.2		
Unknown	33	9.5	0	0	0	0		
Nuclear pAKT							0.38	< 0.001
Negative	210	60.5	133	78.2	59	41.0		
Positive	125	36.0	37	21.8	84	58.3		
Unknown	12	3.5	0	0	1	0.7		
Cytoplasmic pAKT							0.01	0.915
Negative	182	52.4	89	52.4	74	51.4		
Positive	153	44.1	81	47.6	69	47.9		
Unknown	12	3.5	0	0	1	0.7		
Nuclear pMAPK							0.26	< 0.001
Negative	181	52.2	112	65.9	57	39.6		
Positive	148	42.7	58	34.1	86	59.7		
Unknown	18	5.2	0	0	1	0.7		
Cytoplasmic pMAPK							0.27	< 0.001
Negative	226	65.1	134	78.8	77	53.5		
Positive	103	29.7	36	21.2	66	45.8		
Unknown	18	5.2	0	0	1	0.7		

Note: 33 patients were missing nuclear NF1 status.
ρ: Spearman's correlation.

Table 4.8: Association between pAKT, pMAPK and NF1 expression (cytoplasmic only) (using a cut-off of 10% of cells staining positive)

Parameter	Total (N=347) Number	%	Cytoplasmic NF1 negative (N=30) Number	%	Cytoplasmic NF1 positive (N=284) Number	%	ρ	p-value
Cytoplasmic pAKT							0.05	0.361
Negative	182	52.4	18	60.0	145	51.1		
Positive	153	44.1	12	40.0	138	48.6		
Unknown	12	3.5	0	0	1	0.4		
Cytoplasmic pMAPK							0.16	0.006
Negative	226	65.1	27	90.0	184	64.8		
Positive	103	29.7	3	10.0	99	34.9		
Unknown	18	5.2	0	0	1	0.4		

Note: 33 patients were missing cytoplasmic NF1 status.
ρ: Spearman's correlation.

Validation Set

To validate the preliminary finding that positive expression of neurofibromin in both nucleus and cytoplasm was associated with better outcomes, immunohistochemical staining with the same neurofibromin antibody was performed on TMAs with a total of 681 triple negative breast cancers diagnosed over the period 1993 to 2011. The clinicopathological characteristics of this validation set are tabulated in Table 4.9, with the characteristics and neurofibromin staining of the triple negative subset from the discovery set side-by-side for comparison. Overall the characteristics of the 2 cohorts appear similar and comparable.

While standard prognostic factors such as tumour grade, stage and the presence of lymphovascular invasion were significant predictors of relapse free survival in this cohort (Table 4.10), there was no correlation of neurofibromin status with relapse status when using the same combined expression definition as in the discovery set (Table 4.11). We failed to find any significant association of relapse free survival with neurofibromin status even when analysed via various definitions, looking at nuclear expression versus cytoplasmic expression individually with different cutoffs, and using H-scores (Table 4.11). The relapse-free survival and overall survival curves of the validation cohort using the same definition as the discovery cohort (staining in at least 10% of the cells in the nucleus and at least 10% in the cytoplasm) are shown in Figures 4.5 and 4.6.

Table 4.9: Clinicopathological characteristics (including neurofibromin staining results) of triple negative validation cohort, and the triple negative subset of the discovery cohort for comparison.

Variable	Validation cohort		Discovery cohort (TNBC)	
	Number	%	Number	%
Total number of patients	681	100.0	95	100.0
Age at diagnosis, years				
Median	52		54	
Range	25 - 89		33-86	
Race				
Chinese	569	83.6	80	84.2
Indian	38	5.6	5	5.2
Malay	54	7.9	9	9.5
Others	20	2.9	1	1.1
Tumour size, mm				
Median	30		30	
Range	2 - 200		2-200	
Unknown	20	2.9	0	
Tumour grade				
1	18	2.6	6	6.3
2	132	19.4	27	28.4
3	529	77.7	62	65.3
Unknown	2	0.3	0	
Histologic subtype				
IDC	627	92.1	88	92.6
ILC	15	2.2	2	2.1
IDC&ILC	1	0.2	0	0
Metaplastic	14	2.1	1	1.1
Medullary	18	2.6	2	2.1
Adenoid cystic	2	0.3	0	0
Papillary	4	0.6	0	0
Mucinous	0	0.0	1	1.1
Lymphovascular invasion				
Absent	474	69.6	70	73.7
Present	200	29.4	25	26.3
Unknown (blank)	7	1.0	0	0
Nodal stage				
0	327	48.0	53	55.8
1	140	20.6	16	16.8
2	80	11.8	16	16.8
3	47	6.9	7	7.4
Unknown	87	12.8	3	3.2

Variable (continued)	Validation cohort Number	%	Discovery cohort (TNBC) Number	%
Nuclear NF1 Total +%				
Median	0		0	
Range	0 - 100		0-95	
Unknown	71	10.4	4	4.2
Cytoplasmic NF1 Total +%				
Median	30		20	
Range	0 - 95		0-90	
Unknown	71	10.4	4	4.2
Nuclear NF1 H-score				
Median	0		0	
Range	0 - 160		0-160	
Unknown	71	10.4	4	4.2
Cytoplasmic NF1 H-score				
Median	30		30	
Range	0 - 140		0-140	
Unknown	71	10.4	4	4.2

Table 4.10: Univariable analysis of relapse-free survival in validation cohort - demographics and baseline clinical characteristics

	No. of events / patients	5-year relapse rate, % (95% CI)	p-value[1]	Hazard ratio (95% CI)	p-value[2]
All patients	251 / 660#	33.3 (29.7 to 37.1)	NA	NA	NA
Tumour size, cm	243 / 642	NA	NA	1.14 (1.10 to 1.18)	<0.001
Tumour grade			0.6		
1	5 / 18	23.0 (9.3 to 50.3)		1	
2	53 / 128	31.1 (23.7 to 40.2)		1.61 (0.64 to 4.02)	0.3
3	192 / 512	34.1 (30.0 to 38.5)		1.61 (0.66 to 3.91)	0.3
Nodal stage			<0.001		
0	83 / 324	20.4 (16.3 to 25.4)		1	
1	54 / 135	33.0 (25.5 to 41.9)		1.76 (1.25 to 2.47)	0.001
2	47 / 77	54.5 (43.5 to 66.2)		3.26 (2.28 to 4.66)	<0.001
3	35 / 45	77.2 (63.5 to 88.5)		6.53 (4.38 to 9.74)	<0.001
Lymphovascular invasion			<0.001		
Absent	147 / 459	27.4 (23.5 to 31.9)		1	
Present	102 / 194	47.3 (40.3 to 54.8)		2.12 (1.65 to 2.73)	<0.001

[1] P-value calculated using Log-rank test
[2] P-value calculated using Wald test (from Cox model)
Date of last follow up not available for 21 patients; NA: Not applicable.

Table 4.11: Univariable analysis of relapse-free survival in validation cohort with neurofibromin status

	No. of events / patients	5-year relapse rate, % (95% CI)	p-value[1]	Hazard ratio (95% CI)	p-value[2]
Total +% using cut-off of 10%					
Combined NF1 expression			1.0		
Negative	146 / 380	33.9 (29.2 to 39.0)		1	
Positive	80 / 214	32.4 (26.4 to 39.3)		1.00 (0.76 to 1.31)	1.0
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.97 (0.63 to 1.48)	0.9
Nuclear NF1			1.0		
<10%	142 / 369	34.0 (29.3 to 39.2)		1	
≥10%	84 / 225	32.3 (26.5 to 39.1)		0.99 (0.76 to 1.30)	1.0
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.97 (0.63 to 1.48)	0.9
Cytoplasmic NF1			0.2		
<10%	23 / 49	44.1 (31.0 to 59.8)		1	
≥10%	203 / 545	32.4 (28.5 to 36.6)		0.74 (0.48 to 1.15)	0.2
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.74 (0.42 to 1.31)	0.3
Total +% using cut-off of 1%					
Combined NF1 expression			0.6		
Negative	133 / 338	34.6 (29.7 to 40.1)		1	
Positive	93 / 256	31.7 (26.2 to 38.0)		0.94 (0.72 to 1.23)	0.6
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.95 (0.62 to 1.45)	0.8
Nuclear NF1			0.6		
≥1%	94 / 259	31.8 (26.3 to 38.0)		0.93 (0.72 to 1.22)	0.6
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.94 (0.61 to 1.45)	0.8
Cytoplasmic NF1			0.7		
0%	9 / 19	42.6 (24.0 to 67.5)		1	
≥1%	217 / 575	33.0 (29.2 to 37.1)		0.87 (0.45 to 1.69)	0.7
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.85 (0.39 to 1.81)	0.7
H-score categorised into 3 groups					
Nuclear NF1			0.5		
< 50	190 / 502	32.5 (28.5 to 36.9)		1	
50 – 99	27 / 70	36.8 (26.6 to 49.5)		1.14 (0.76 to 1.71)	0.5
≥ 100	9 / 22	44.6 (23.8 to 72.3)		1.45 (0.74 to 2.83)	0.3
Unknown	25 / 66	32.6 (22.6 to 45.5)		1.00 (0.66 to 1.51)	1.0
Cytoplasmic NF1			0.6		
< 50	157 / 422	32.8 (28.4 to 37.7)		1	
50 – 99	59 / 152	33.2 (26.1 to 41.5)		1.00 (0.74 to 1.35)	1.0
≥ 100	10 / 20	45.0 (26.5 to 68.7)		1.40 (0.74 to 2.65)	0.3
Unknown	25 / 66	32.6 (22.6 to 45.5)		0.98 (0.64 to 1.50)	0.9

[1] P-value calculated using Log-rank test, excluding the category "Unknown"
[2] P-value calculated using Wald test (from Cox model)

Figure 4.5: Kaplan Meier plot of relapse-free survival according to expression of neurofibromin (staining in at least 10% of the cells in the nucleus and at least 10% in the cytoplasm to be “positive”) in triple negative validation cohort.

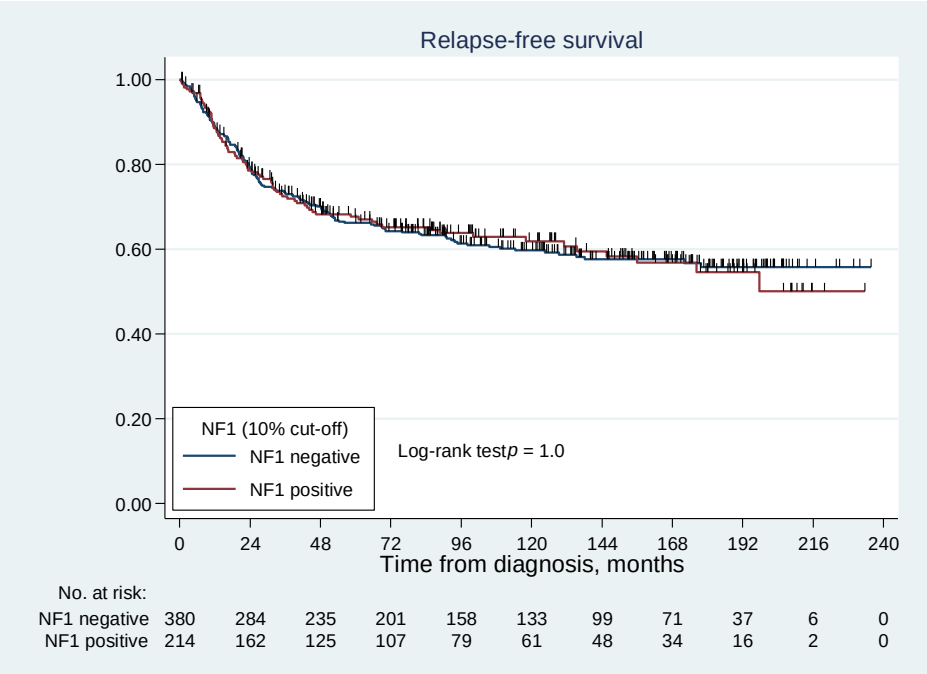
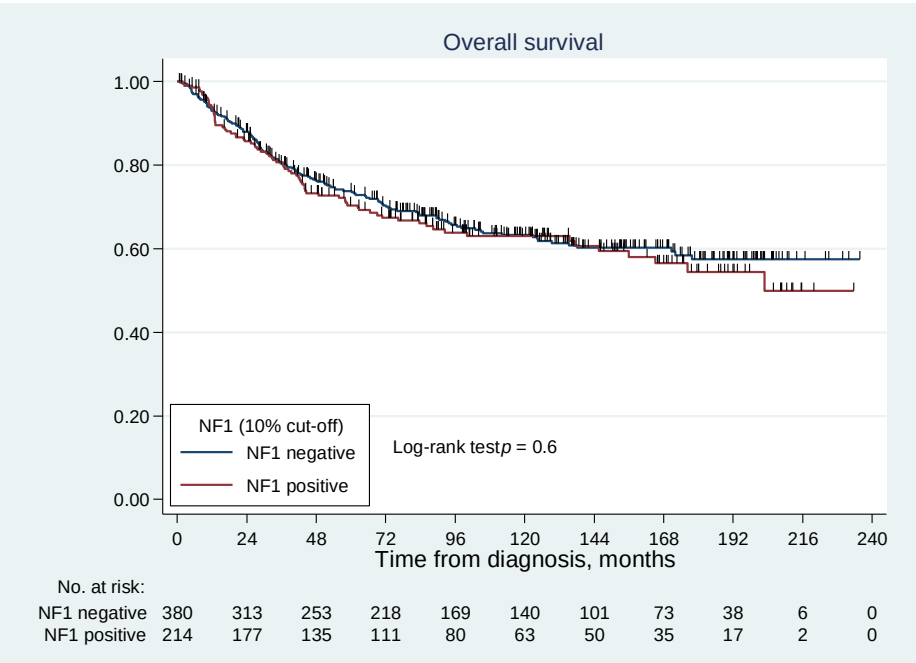


Figure 4.6: Kaplan Meier plot of overall survival according to expression of neurofibromin (staining in at least 10% of the cells in the nucleus and at least 10% in the cytoplasm to be “positive”) in triple negative validation cohort.



4.5 Discussion

With the increasing evidence of the critical role that *NF1* plays in breast cancer and other tumours, we had postulated that loss of expression of neurofibromin would be associated with inferior survival outcomes. We sought to evaluate the deficiency of neurofibromin using immunohistochemistry on TMAs of sporadic breast cancers, given that loss of *NF1* appears to be more common than mutations, and expression of neurofibromin may be influenced by epigenetic or proteasomal mechanisms [1].

Data on the use of neurofibromin antibody is relatively limited. Apart from the study which reported no differences in expression of neurofibromin in 22 sporadic breast cancers, 18 benign lesions and 6 normal breast tissue specimens [7], the same antibody to neurofibromin (ab30325, 1:300 dilution; Abcam, Cambridge, UK) has also been used in a study on melanomas by Maertens et al [8]. It reported complete absence of neurofibromin in 15-18% of sporadic melanomas which had developed resistance to BRAF inhibitors. Loss of neurofibromin was also associated with activation of pAkt and pERK(or pMAPK) in Western blot, usint the same antibodies that we used for this study. A different study assessed the expression of neurofibromin in lung cancers via Western Blotting with antibody to NF1 (#A300-140) from Bethyl. Protein expression was lower in *EGFR*-mutant lung carcinomas that had developed to EGFR inhibitors, compared to treatment-naïve tumours which remained sensitive to erlotinib [14].

Taking into consideration that many tumour suppressors, including neurofibromin, shuttle between the nucleus and the cytoplasm [11], we adopted a definition of neurofibromin positivity which requires its presence in at least 10% of the cells in the nucleus and at least 10% in the cytoplasm as well, though the nuclear and cytoplasmic staining do not have to be in the same cells. Neurofibromin regulates the Ras/PI3K/MAPK proliferation pathway in the cytoplasm, but is also actively transported to the nucleus [11]. Within the nucleus, it may affect the expression of other genes, potentially suppressing epithelial mesenchymal transition (EMT) [1,15]. In hormone receptor positive breast cancer, a very recent study elucidated an additional role of neurofibromin in co-repressing the estrogen receptor (ER)[16]. *NF1* inactivation was reported to enhance ligand-dependent ER transcriptional activity. *NF1*-silencing

can turn tamoxifen, a selective estrogen receptor modulator used to breast cancer, into an agonist instead, making the cells hypersensitive to estradiol [16].

In the discovery cohort, we found that “negative” expression of neurofibromin was associated with adverse prognostic features such as high tumour grade (p<0.001), hormone receptor negativity (p<0.001), lymph node positivity (p=0.041) and larger tumour size (p=0.031). It was also an independent prognostic factor for triple negative breast cancers in multivariable analysis for both relapse-free survival, as well as overall survival (hazard ratios 3.33, p=0.011 and 2.94, p=0.026 respectively). However, validation in a larger series of triple negative breast cancers did not support this initial finding.

This discrepancy may be due to the relatively small sample size of different subtypes in the discovery cohort, or due to limitations of immunohistochemical staining on archived TMAs. We also did not find any correlation of lack of neurofibromin with expression of pAkt and pMAPK as hypothesized initially. Staining with phospho-antibodies may not be reliable in old archived FFPE specimens. Immunohistochemical staining with neurofibromin antibody may also be suboptimal as the current antibodies available are based on detection of a specific epitope, and may not be able to distinguish between the normal and mutant neurofibromin proteins, especially in the case of missense mutations for such a large protein. Deletions and frameshift changes downstream to the region of the epitope for the antibody resulting in truncated neurofibromin will also not be detected. For example, the antibody for neurofibromin (ab30325, 1:300 dilution; Abcam, Cambridge, UK) in this study only detects a small epitope (amino acids 27-41) close to the N-terminus in the neurofibromin protein which contains 2,818 amino acids [17]; this antibody is no longer in production.

Correlation with NF1 genomic status was not performed in this study, due to challenges with DNA from FFPE specimens, as well as limitation with time and resources. Another possibility is that with the heterogeneity of the genomic landscape of breast cancers, the tumours are likely to harbor aberrations in other cancer-related genes, which may have similar effect to the presence of inactivating *NF1* mutations or losses, confounding the outcome associations. Other possible reasons for the discrepancy in findings include the different time period of the discovery (2000-2002)

and validation (1993-2011) cohorts. Adjuvant treatment details could not be retrieved for all patients due to issues with access to old clinical records, but treatment changes over the time period include possibly the use of more chemotherapy and incorporation of taxanes in addition to anthracyclines since early 2000s. The treatment variables are unlikely to affect the results to any significant extent as the standard prognostic variables of tumour size, grade and stage have remained robust in predicting survival outcomes in both cohorts.

4.6 Conclusions

While the final results of this TMA study did not support our initial hypothesis that loss of expression of neurofibromin in sporadic breast cancers would be associated with overexpression of pAkt and pMAPK, leading to worse outcomes, this may be related to the limitations of using immunohistochemistry to evaluate the function of complex proteins. It may be worth repeating the study with the use of imaging analysis software and a different neurofibromin polyclonal antibody raised against a peptide at the C-terminus, if there is one available. However, novel proteomic platforms such as mass spectrometry[18] may potentially be more promising for elucidating the effects of abnormalities in, or loss of neurofibromin in sporadic breast cancers.

4.7 References

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Chapter 5

Elucidating Therapeutic Molecular Targets in Premenopausal Asian Women with Recurrent Breast Cancers

5.1 Abstract

Breast cancer is an increasing problem in Asia, with a higher proportion of premenopausal patients who are at higher risk of recurrence. Targeted sequencing was performed on DNA extracted from primary tumour specimens of 63 premenopausal Asian patients who relapsed after initial diagnosis of non-metastatic breast cancer. The most prevalent alterations included: *TP53* (65%); *PIK3CA* (32%); *GATA3* (29%); *ERBB2* (27%); *MYC* (25%); *KMT2C* (21%); *MCL1* (17%); *PRKDC, TPR, BRIP1* (14%); *MDM4, PCDH15, PRKAR1A, CDKN1B* (13%); *CCND1, KMT2D, STK11* and *MLH1* (11%). *NF1* was mutated in 2% of the tumours; there were limitations with detecting loss of *NF1* in FFPE specimens. Sixty of the 63 patients (95%) had at least one genetic alteration in a signaling pathway related to cell cycle or p53 signaling. The presence of *MCL1* amplification, HIF-1-alpha transcription factor network pathway alterations and direct p53 effectors pathway alterations were independent predictors of inferior overall survival from initial diagnosis. Comparison with non-Asian premenopausal tumours in The Cancer Genome Atlas (TCGA) revealed a higher prevalence of *TP53* mutations among HER2-positive cancers, and more frequent *TP53, TET2* and *CDK12* mutations among hormone receptor positive HER2-negative cancers in our cohort. Given the limited number of non-Asian premenopausal breast cancers that had relapsed in TCGA, we compared the frequency of mutations in our cohort with 43 premenopausal specimens from both TCGA and International Cancer Genome Consortium (ICGC) that had relapsed. There was a trend towards higher prevalence of *TP53* mutations in our cohort. Certain genomic aberrations may be enriched in tumours of poor-prognosis premenopausal Asian breast cancers. The development of novel therapies targeting these aberrations merits further research.

5.2 Introduction

Breast cancer is an increasing health problem in East Asia where the incidence of breast cancer has been rising dramatically over the past few decades. In contrast to the trends in the United States which report stable or decreasing breast cancer incidence rates among Chinese Americans and non-Hispanic whites over the period 1990-2008, the breast cancer rates have been increasing in several Asian countries, with net drift at 2.67% per year for women in Singapore, and as high as 5.64% per year for women from rural China [1]. For the younger age groups, the incidence of breast cancer in several Asian countries, such as Singapore and South Korea, has even surpassed that in the United States [1,2].

The recent increase in breast cancer rates cannot be solely attributed to the effects of screening and improved data capture. The adoption of a Westernised lifestyle in recent generations has been suggested as a major cause of this trend. Hormonal risk factors such as earlier age at menarche, low parity, delayed age at first birth, rising body mass index and dietary factors with increased consumption of fat and animal-source products, have been implicated [2-4]. In addition, the progressive urbanization and industrialization of East Asia has been suggested to result in increasing exposure of women to environmental pollutants with oestrogenic effects, and this may contribute further to the increasing incidence of breast cancer [4].

There are distinct ethnic differences in the biology of certain cancers such as lung adenocarcinoma, where the frequency of mutations in *EGFR* (epidermal growth factor receptor) is significantly higher in East Asians compared to Western populations [5]. To date, such differences have not been reported in breast cancer. However compared with Western countries, the age of diagnosis of breast cancer is generally younger in East Asia [2,4,6]. Over 40% of breast cancers in Asia are diagnosed in women under 50 years of age, compared to approximately 20% in Western countries [6]. Younger women with early breast cancer are at higher risk of relapse and death from breast cancer; this may be related to differences in tumour and/or host biology [7]. Comparison of breast cancers from young and elderly women in The Cancer Genome Atlas (TCGA) revealed an association of *GATA3* mutations and chr6q27 deletions with younger age,

and higher expression of gene signatures related to proliferation, stem cell features and endocrine resistance [8].

Given the limited data on Asian breast cancers, and the higher prevalence in premenopausal patients, it is critical to elucidate the genomic landscape in premenopausal Asian patients who relapse after initial diagnosis of non-metastatic breast cancer. The main objective of this study is to identify actionable genomic aberrations in the primary breast cancers from premenopausal Asian patients who subsequently relapse. We also aimed to identify the genomic aberrations associated with inferior survival outcomes, and to compare the frequency of these genomic aberrations with non-Asian premenopausal tumours in publicly available databases such as TCGA and International Cancer Genome Consortium (ICGC).

5.3 Materials and Methods

Patients and samples

Women who were premenopausal on initial diagnosis of breast cancer and relapsed subsequently were identified during outpatient clinic visits, inpatient hospital admissions, or from the institutional database at the National Cancer Centre Singapore and the Asan Medical Centre, South Korea. Demographic data, histopathological features, treatment details and patient outcome (time to relapse and overall survival) were obtained from medical records. The definitions of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) positivity in this study were based on the latest recommendations by the American Society of Clinical Oncology and the College of American Pathologists [9,10]. The study design was conceived by the candidate, and was approved by SingHealth Centralised Institutional Review Board and Asan Medical Center Institutional Review Board in the respective institutions. This research was conducted in accordance with all relevant guidelines and procedures, with signed informed consent obtained from patients over 2014-2016, and waiver of consent from deceased patients as granted by the local ethics committees.

Next Generation Sequencing (NGS) analysis of somatic mutations, indels and copy-number calls

DNA extraction, QC, library preparation and pipeline analysis were performed by collaborators from Oncology Next-Generation Diagnostics, Novartis Institutes for Biomedical Research, Cambridge, USA. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) sections of the initial breast cancer primary with at least 50% tumour percentage from 110 patients, using Promega Maxwell DNA purification kit as per manufacturer's instructions. DNA libraries were generated using the TruSeq Nano Library Preparation kit (Illumina). Hybridization capture to a customised Agilent SureSelectXT panel was used to enrich coding regions from 567 cancer-related genes. The analysis cohort consisted of 63 unique samples that passed quality control metrics for sequencing on a HiSeq-2500 with average coverage of at least 300X. Other quality control requirements included a mean bait coverage > 200, and the Picard HsMetrics parameters of GC_DROPOUT < 20 and AT_DROPOUT < 20. The average coverage achieved was 630X, average on-target rate was ~ 80% and the average duplication rate was 26.5%. The NGDx PanCancer version 2 panel interrogates the entire coding sequence of 567 cancer-related genes plus select introns from 57 genes often rearranged or altered in solid tumor cancers (Supplementary Table S5.1).

Sequencing data was processed as follows: Sequence reads were aligned with BWA-MEM to the reference human genome (build hg19) (https://arxiv.org/abs/1303.3997). Next, PCR duplicates were marked with Picard (http://broadinstitute.github.io/picard/) and the Genome Analysis ToolKit (GATK) was used for local realignment and base quality score recalibration [11,12]. Single nucleotide variants (SNVs) were called using MuTect [13], indels using Pindel [14] and copy number was called using PureCN [15]. SNVs and indels were annotated with dbSNP v146 [16], COSMIC v70 [17] and for various other databases using the SnpEff tool [18].

SNVs and indels were filtered for germline variants and artifacts using a pool of normal control samples and an extensive set of filters using the dbSNP and 1000 genomes databases. SNV were considered as sequencing artifacts if they were observed in at least 2 of 50 normal samples, or occurred within simple repeats or segmental duplications in the UC Santa Cruz reference genome annotation. SNV were considered as germline variants if they matched one of the following 3 criteria: (1) found in at least 2 samples in the Exome Sequencing Project database; (2) annotated as common by dbSNP (>5% minor allele fraction in 1 or more populations, as

determined by the G5 flag in dbSNP); (3) identified in the 1000 Genomes Project. Putative germline variants could be rescued if they were annotated in the COSMIC database. Otherwise, germline variants were excluded from further analyses. Non-silent variants with a minimum of 5 supporting reads and total read coverage ≥ 50X were retained. For indels, the minimum threshold was set at 4 reads. Indel length size was capped at 100bp.

Allele-specific copy number was obtained from coverage data for probe intervals. Coverage was first normalized for GC-bias. The purity, ploidy and copy number were jointly estimated for each sample using PureCN [15]. For coverage normalization, PureCN uses a pool of normal samples to determine a 'best-match' set of normals using Principal Component Analysis. Probe-level copy number values are averaged into per-gene values before reporting. We also conducted pathway level analysis of 16 most relevant pathways in cancer development and progression (Supplementary Table S5.2).

Genomic analysis of TCGA and ICGC samples

TCGA data was obtained from the cBioPortal and included the most comprehensive set of cases from the "TCGA Provisional" dataset (http://www.cbioportal.org/study?id=brca_tcga). The ICGC data included the BRCA-EU study and was obtained from the ICGC Portal (https://dcc.icgc.org/projects/BRCA-EU). Since this was a whole genome study, we only retained and analysed mutations in the 567 cancer-related genes present in the PanCancer panel. All intergenic, intronic, upstream, downstream and other silent mutations or others of no significance were discarded for comparison purposes. In addition, single nucleotide calls made using the CaVEMan and indels called by Pindel alone were retained in accordance with the reporting of genomic data [19]. Clinical characteristics of the patients were picked up from the Supplementary Material in the publications [19,20].

Statistical Analyses

For each gene, a Fisher exact test was used to assess differences in the population frequencies of mutations (SNV and indels) in this cohort, versus pre-menopausal, non-Asian breast cancer subjects from TCGA and/or ICGC. The Benjamini-Hochberg

method of computing the False Discovery Rate was used to adjust for multiple hypothesis testing.

Overall survival (OS) distributions were estimated using the Kaplan-Meier method, and differences in survival from initial diagnosis were assessed with log-rank tests. Adjusting for age at diagnosis and breast cancer subtype, two multivariable Cox proportional hazards regression models (one to assess the associations of genetic alteration status with OS, another to assess the associations of pathway alterations with OS) were estimated. For the model assessing genetic alteration associations, variable selection was performed via best subsets selection using the Akaike Information Criterion (AIC), constrained with the compulsory inclusion of age and subtype. Thirteen of the most commonly altered genes (alteration frequency of 13% and above) were selected for this multivariable analysis of single gene alterations. For the model assessing pathway alteration associations, due to high pairwise correlations among pathway alterations and issues of multicollinearity, variable selection was performed via regularized coefficient shrinkage using the LASSO (Least Absolute Shrinkage and Selection Operator) technique, leaving age and subtype unpenalized. These analyses were performed using STATA 15.0.

5.4 Results

Clinical and Pathological Characteristics

A total of 110 patients were identified over the study period from September 2014 to May 2016 to have relapsed from breast cancer and had been premenopausal at initial diagnosis. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) sections of the initial breast cancer primary from these patients, of which 63 unique samples passed quality control. Hence the patient population with sequencing data consists of 63 women who were premenopausal at initial diagnosis from 2008 to 2015 (Table 5.1). The median age at diagnosis was 42 years (range 25-49 years). Majority of the cases were stage 2 (41.3%) or stage 3 (52.4%) at diagnosis. The most common immunohistochemical subtype was “luminal” (defined as ER and/or PR+ HER2- in this study) at 42.9%, followed by HER2-overexpressing subtype (regardless of hormone receptor status) at 33.3%, while triple negative cases comprised 23.8% of patients in the cohort. The median time to relapse was 23 months (range 6-150 months). The

median OS was 59 months (95% C.I. 45-79 months) after initial diagnosis, and 25 months (95% C.I. 20-27 months) after relapse.

Prevalence of genomic alterations

Among the 63 cases with sequencing data, a total of 406 SNVs and 52 indels were found (Figure 5.1, Supplementary Table S5.3). The most prevalent mutations and amplifications in oncogenes included: *PIK3CA* (32%); *GATA3* (29%); *ERBB2* (27%); *MYC* (25%); *MCL1* (17%); *PRKDC* (14%); *MDM4* (13%); and *CCND1* (11%). While *NF1* was altered in 6% (4/63) of the tumours – only one harboured a mutation (1/63=1.6%), with amplification detected in 3 other tumours. Detecting loss of *NF1* was not feasible with the DNA from FFPE specimens. The most prevalent alterations in tumor suppressor genes included: *TP53* (65%); *KMT2C* (21%); *PCDH15* (13%); *KMT2D* (11%); *STK11* (11%); and *MLH1* (11%). Sixty of the 63 patients (95%) had at least one genetic alteration in a signaling pathway related to cell cycle or p53 signaling (Supplementary Table S5.2).

Table 5.1: Patient and Primary Tumour Characteristics

Characteristics	Age at diagnosis (years)			Total (*n*=63)
	<35 (*n*=16)	35-44 (*n*=28)	≥45 (*n*=19)	
	No. (%)	No. (%)	No. (%)	No. (%)
Age in years, median (range)				42 (25 – 49)
Follow-up in months from initial diagnosis, median (range)				44 (8 – 168)
Ethnicity				
Chinese	6 (37.5)	13 (46.4)	9 (47.4)	28 (44.4)
Korean	7 (43.8)	10 (35.7)	5 (26.3)	22 (34.9)
Malay	0	3 (10.7)	5 (26.3)	8 (12.7)
Indian	2 (12.5)	0	0	2 (3.2)
Others	1 (6.3)	2 (7.1)	0	3 (4.8)
AJCC stage				
0	0	0	1 (5.3)	1 (1.6)
I	0	1 (3.6)	1 (5.3)	2 (3.2)
II	5 (31.3)	11 (39.3)	10 (52.6)	26 (41.3)
III	10 (62.5)	16 (57.1)	7 (36.8)	33 (52.4)
Unknown (TxN1M0)	1 (6.3)	0	0	1 (1.6)
Grade				
1	0	0	0	0
2	6 (37.5)	11 (39.3)	6 (31.6)	23 (36.5)
3	10 (62.5)	16 (57.1)	13 (68.4)	39 (61.9)
Unknown	0	1 (3.6)	0	1 (1.6)
ER status at diagnosis				
Positive	9 (56.3)	19 (67.9)	8 (42.1)	36 (57.1)
Negative	7 (43.8)	9 (32.1)	11 (57.9)	27 (42.9)
PR status at diagnosis				
Positive	7 (43.8)	13 (46.4)	7 (36.8)	27 (42.9)
Negative	9 (56.3)	15 (53.6)	12 (63.2)	36 (57.1)
HER2 status at diagnosis				
Positive	4 (25.0)	11 (39.3)	6 (31.6)	21 (33.3)
Negative	12 (75.0)	17 (60.7)	13 (68.4)	42 (66.7)
Immunohistochemical subtype				
“Luminal” (ER and/or PR+, HER2-)	7 (43.8)	13 (46.4)	7 (36.8)	27 (42.9)
HER2-overexpressing (regardless of ER/PR)	4 (25.0)	11 (39.3)	6 (31.6)	21 (33.4)
Triple negative (ER-, PR-, HER2-)	5 (31.3)	4 (14.3)	6 (31.6)	15 (23.8)
Histology				
Ductal	15 (93.8)	25 (89.3)	15 (78.9)	55 (87.3)
Lobular	0	0	2 (10.5)	2 (3.2)
Others	1 (6.3)	3 (10.8)	2 (10.6)	6 (9.6)
Adjuvant/neoadjuvant chemotherapy				
No	0	3 (10.7)	4 (21.1)	7 (11.1)
Yes	16 (100)	25 (89.3)	15 (78.9)	56 (88.9)
Adjuvant/neoadjuvant anti-HER2 therapy (among HER2+)				
No	0	1 (9.1)	1 (16.7)	2 (9.5)
Yes	4 (100)	10 (90.9)	5 (83.3)	19 (90.5)
Adjuvant/neoadjuvant endocrine therapy (among ER+ and/or PR+)				
No	0	3 (15.8)	2 (22.2)	5 (13.5)
Yes	9 (100)	16 (84.2)	7 (77.8)	32 (86.5)

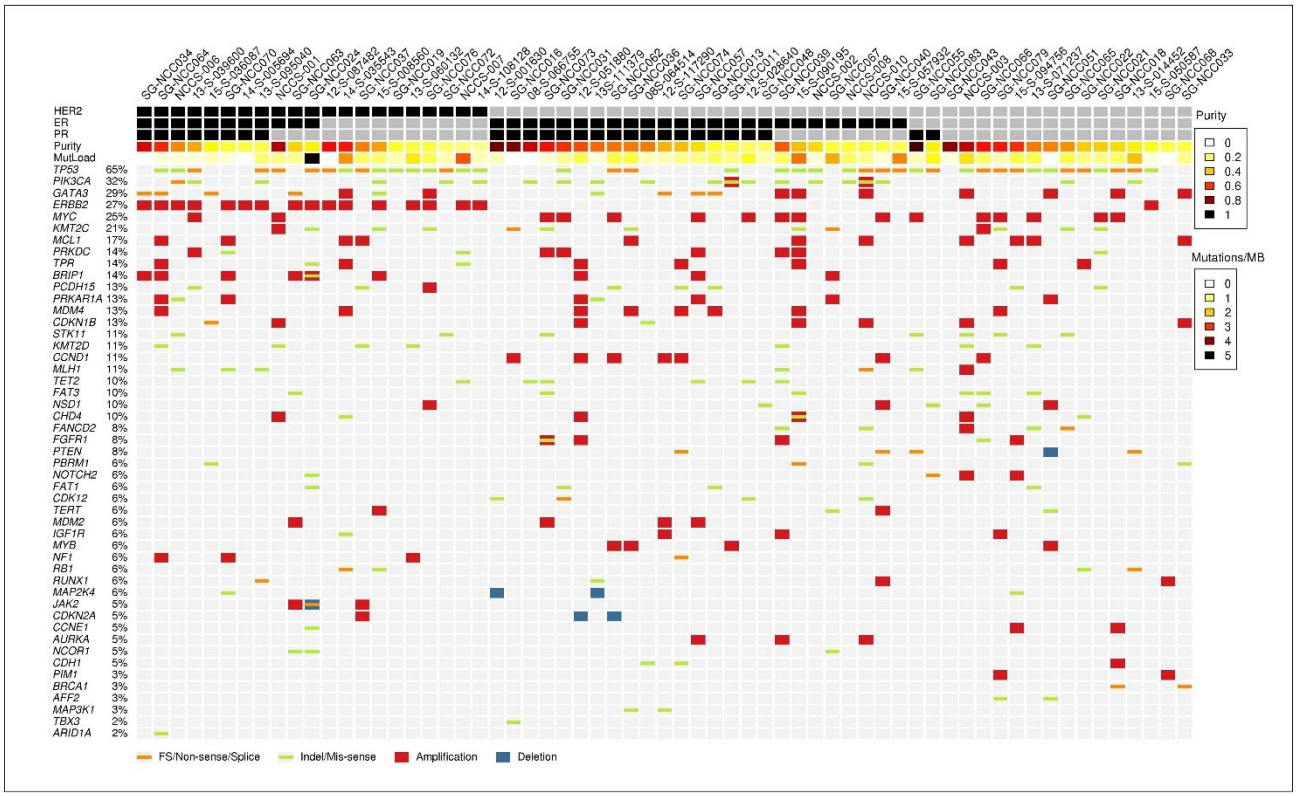

Figure 5.1: Genomic profile of 63 tumor samples ordered by receptor status: HER2+ (*n* = 21), hormone receptor (HR)+ and HER2- (*n* = 27) and Triple Negative (*n* = 15), and purity.
The figure lists the prevalence of mutations, indels and copy number variations for recurrently mutated genes in breast cancer and the mutation load of each sample. Purity and mutation load are indicated for each sample. The darker the shade, the higher the value.

Association between clinical, molecular characteristics and survival outcomes

From multivariable analysis where the resultant model incorporated age, immunohistochemical subtype as well as other clinicopathologic characteristics including the most common gene alterations, age (HR 1.10 per year increase; 95% CI 1.03-1.17; *p*=0.006) and presence of *MCL1* amplification (HR 4.24; 95% CI 1.62-11.07; *p*=0.003) were found to be independent predictors of OS from initial diagnosis (Table 5.2).

Given that mutations in different genes can compromise a particular pathway and this may provide a better prediction of outcomes compared to alterations in individual genes, we also tested for associations between pathway alterations and OS (Table 5.3). From multivariable analysis, only age (HR 1.09 per year increase; 95% CI 1.02-1.17; *p*=0.008), presence of alterations in the HIF-1-alpha transcription factor network

pathway, which includes *MCL1* among other genes (HR 2.57; 95% CI1.18-5.60; *p*=0.017), and presence of alterations in direct p53 effectors (HR 3.61; 95% CI 1.08-12.01; p=0.036) were predictive of inferior OS from initial diagnosis.

Table 5.2: Multivariable analysis for patient and tumour characteristics, including genetic alterations (all patients, *n*=63) for OS from initial diagnosis. Reduced model is the model with lowest AIC, and inferences should be made from here.

[1] Based on Wald test; [2] Based on likelihood ratio test

	Full model		*Reduced model*	
Characteristic	HR (95% CI)	*P*[1]	HR (95% CI)	*P*[1]
Age (hazard ratio per year increase)	1.11 (1.02 – 1.20)	0.013	1.10 (1.03 – 1.17)	0.006
Immunohistochemical subtype		0.117[2]		0.196[2]
"Luminal"	1	-	1	-
HER2-overexpressing	0.28 (0.08 – 1.00)	0.049	0.52 (0.20 – 1.31)	0.166
Triple negative	0.66 (0.20 – 2.20)	0.498	1.28 (0.51 – 3.20)	0.599
AJCC stage				
0, I, II	1	-	1	-
III	2.07 (0.80 – 5.37)	0.135	1.88 (0.89 – 3.95)	0.097
Grade				
1, 2	1	-	-	-
3	0.63 (0.17 – 2.36)	0.492	-	-
TP53 alteration				
No	1	-	-	-
Yes	2.51 (0.47 – 13.51)	0.284	-	-
PIK3CA alteration				
No	1	-	-	-
Yes	0.67 (0.25 – 1.76)	0.414	-	-
GATA3 alteration				
No	1	-	-	-
Yes	1.11 (0.38 – 3.19)	0.851	-	-
MYC alteration				
No	1	-	-	-
Yes	1.83 (0.63 – 5.31)	0.264	-	-
KMT2C alteration				
No	1	-	-	-
Yes	1.48 (0.51 – 4.28)	0.469	-	-
MCL1 alteration				
No	1	-	1	-
Yes	4.93 (1.29 – 18.79)	0.020	4.24 (1.62 – 11.07)	0.003
PRKDC alteration				
No	1	-	-	-
Yes	0.76 (0.20 – 2.87)	0.691	-	-
TPR alteration				
No	1	-	-	-
Yes	1.60 (0.28 – 9.19)	0.598	-	-
BRIP1 alteration				
No	1	-	-	-
Yes	2.08 (0.21 – 20.75)	0.533	-	-
CDKN1B alteration				
No	1	-	-	-
Yes	1.89 (0.54 – 6.63)	0.319	-	-
PCDH15 alteration				
No	1	-	-	-
Yes	1.56 (0.48 – 5.03)	0.455	-	-
PRKAR1A alteration				
No	1	-	1	-
Yes	0.25 (0.02 – 2.50)	0.237	0.38 (0.11 – 1.27)	0.116
MDM4 alteration				
No	1	-	-	-
Yes	0.39 (0.06 – 2.53)	0.326	-	-

Table 5.3: Multivariable analysis for patient and tumour characteristics, pathway alterations (all patients, *n*=63) for OS from initial diagnosis. Reduced model contains only the variables selected via LASSO regularization, and inferences should be made from here.
[1] Based on Wald test; [2] Based on likelihood ratio test

	Full model		*Reduced model*	
Characteristic	HR (95% CI)	*P*[1]	HR (95% CI)	*P*[1]
Age (hazard ratio per year increase)	1.10 (1.01 – 1.20)	0.026	1.09 (1.02 – 1.17)	0.008
Immunohistochemical subtype		<0.001[2]		0.165[2]
"Luminal"	1	-	1	-
HER2-overexpressing	0.07 (0.01 – 0.31)	0.001	0.48 (0.20 – 1.16)	0.103
Triple negative	3.33 (0.63 – 17.76)	0.158	1.07 (0.42 – 2.72)	0.894
AJCC stage				
0, I, II	1	-	-	-
III	4.29 (1.20 – 15.31)	0.025	-	-
Grade				
1, 2	1	-	-	-
3	1.36 (0.32 – 5.71)	0.675	-	-
Chromatin remodeling by hswi/snf atp-dependent complexes				
No	1	-	-	-
Yes	0.18 (0.02 – 1.45)	0.107	-	-
Role of ERBB2 in signal transduction and oncology				
No	1	-	-	-
Yes	0.41 (0.08 – 1.95)	0.261	-	-
P53 signaling pathway				
No	1	-	-	-
Yes	1.45 (0.06 – 37.74)	0.822	-	-
Cell cycle:g2/m checkpoint				
No	1	-	-	-
Yes	0.38 (0.00 – 48.26)	0.697	-	-
Mapkinase signaling pathway				
No	1	-	-	-
Yes	0.10 (0.02 – 0.47)	0.003	-	-
Mtor signaling pathway				
No	1	-	-	-
Yes	0.56 (0.10 – 3.29)	0.524	-	-
Cell cycle:g1/s checkpoint				
No	1	-	-	-
Yes	0.04 (0.00 – 1.90)	0.104	-	-
C-MYC pathway				
No	1	-	-	-
Yes	0.62 (0.11 – 3.39)	0.579	-	-
Notch signaling pathway				
No	1	-	-	-
Yes	8.76 (1.97 – 38.97)	0.004	-	-
BRCA1 dependent ub ligase activity				
No	1	-	-	-
Yes	0.03 (0.00 – 0.31)	0.002	-	-
PTEN dependent cell cycle arrest and apoptosis				
No	1	-	-	-
Yes	8.39 (1.40 – 50.29)	0.020	-	-
Rb tumor suppressor/checkpoint signaling in response to dna damage				
No	1	-	-	-
Yes	0.11 (0.00 – 3.15)	0.199	-	-

HIF-1-alpha transcription factor network				
No	1	-	1	-
Yes	3.60 (0.80 – 16.17)	0.095	2.57 (1.18 – 5.60)	0.017
IL6-mediated signaling events				
No	1	-	-	-
Yes	0.86 (0.13 – 5.51)	0.870	-	-
E2F transcription factor network				
No	1	-	-	-
Yes	1.93 (0.24 – 15.25)	0.534	-	-
Direct p53 effectors				
No	1	-	1	-
Yes	2358.48 (1.46 – 3810528.00)	0.039	3.61 (1.08 – 12.01)	0.036

Comparison with premenopausal non-Asian breast cancers in TCGA[20]

The prevalence of mutations was compared between this cohort and 167 premenopausal, non-Asian breast cancer patients from TCGA with information on hormone receptor and HER2 status (Figure 5.2). The small number of Asian premenopausal cases in TCGA cohort, with 9 hormone receptor positive, HER2 negative tumours, 5 HER2 positive tumours and no triple negative cancers, precludes any meaningful comparison with our cohort. Given the variability in copy number calling on a number of platforms with varying sample purity levels across different laboratories in TCGA, it is challenging to perform an accurate comparison of copy number alterations with our dataset. Hence comparisons with genomic data were made only for the mutations in the set of 567 genes used in our study.

Comparison of the HER2-positive cases (regardless of hormone receptor status) revealed a significantly higher prevalence of *TP53* (76%) mutations (FDR $q < 0.05$) in our cohort (Figure 5.2A). Among the hormone receptor (HR) positive and HER2 negative cases, there was a significantly higher prevalence of *TP53* (48%), *TET2* (18%) and *CDK12* (15%) mutations (FDR $q < 0.05$) (Figure 5.2B) than TCGA cohort. With the small number of triple negative breast cancer patients, no significant difference in genetic alterations was found (Figure 5.2C).

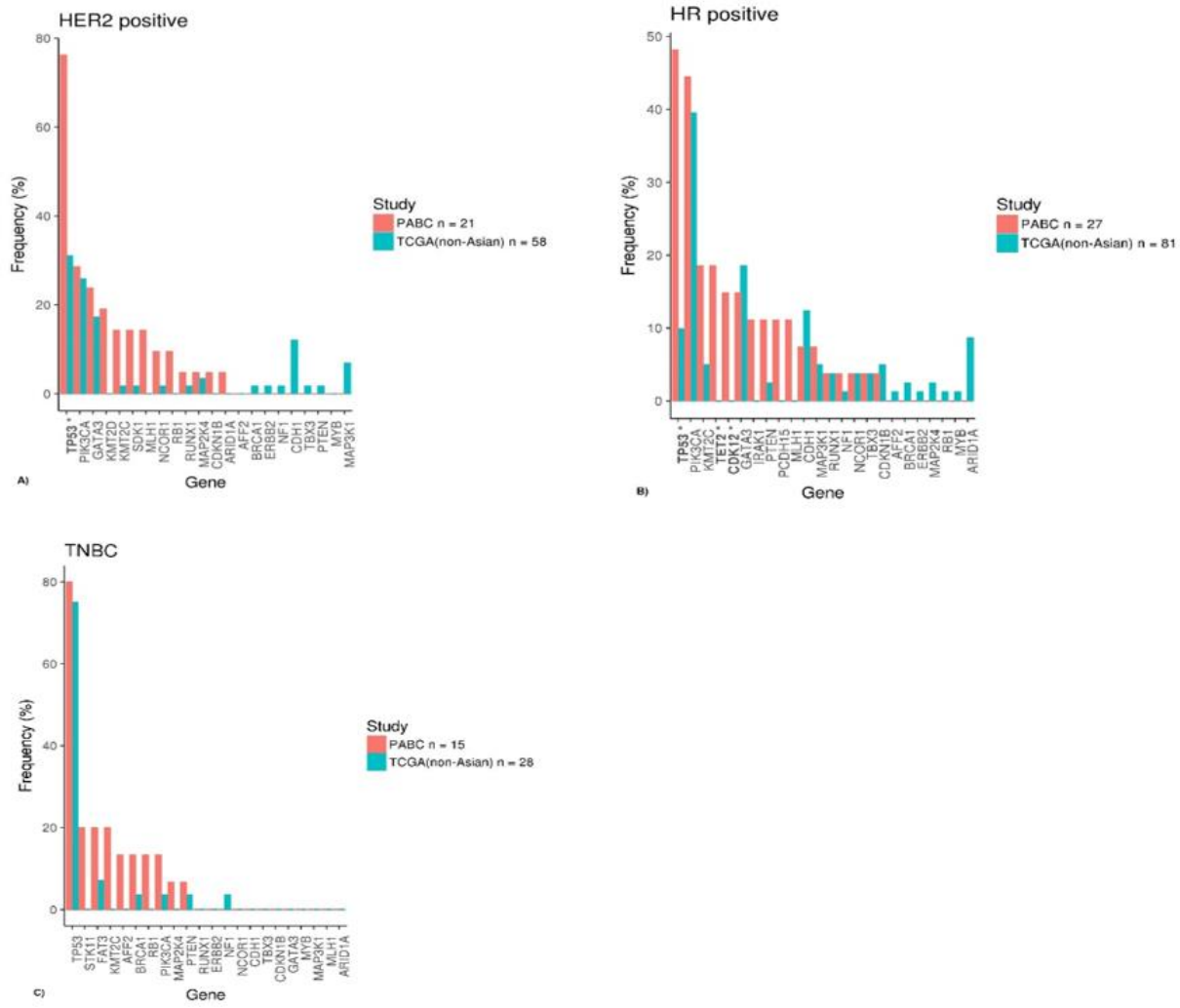

Figure 5.2: Mutation prevalence in our cohort (PABC: Premenopausal Asian Breast Cancers) and comparison to pre-menopausal, non-Asian breast cancer patients in The Cancer Genome Atlas.

Starred genes indicate significant difference from TCGA data (FDR $q < 0.05$). (A) Comparison of 21 HER2-positive samples against 58 non-Asian TCGA samples. (B) Comparison of 27 hormone receptor-positive samples against 81 non-Asian TCGA samples. (C) Comparison of 15 triple negative breast cancers against 28 non-Asian TCGA samples.

<u>Comparison with premenopausal breast cancers in TCGA and ICGC that subsequently relapsed</u>

In order to compare frequently-mutated genes between Asian and non-Asian premenopausal breast cancers, we surveyed 43 premenopausal breast cancer patients who had relapsed from both TCGA and ICGC studies (25 non-Asian premenopausal from TCGA and 18 premenopausal from ICGC-EU) (Figure 5.3) [19,20]. ICGC is currently the only other publicly available dataset with information on recurrence status. Comparison between all patients revealed a higher prevalence of

TP53 mutations (65% versus 35%) in our cohort (Figure 5.3A). However the limited number of cases in both cohorts did not result in statistical significance.

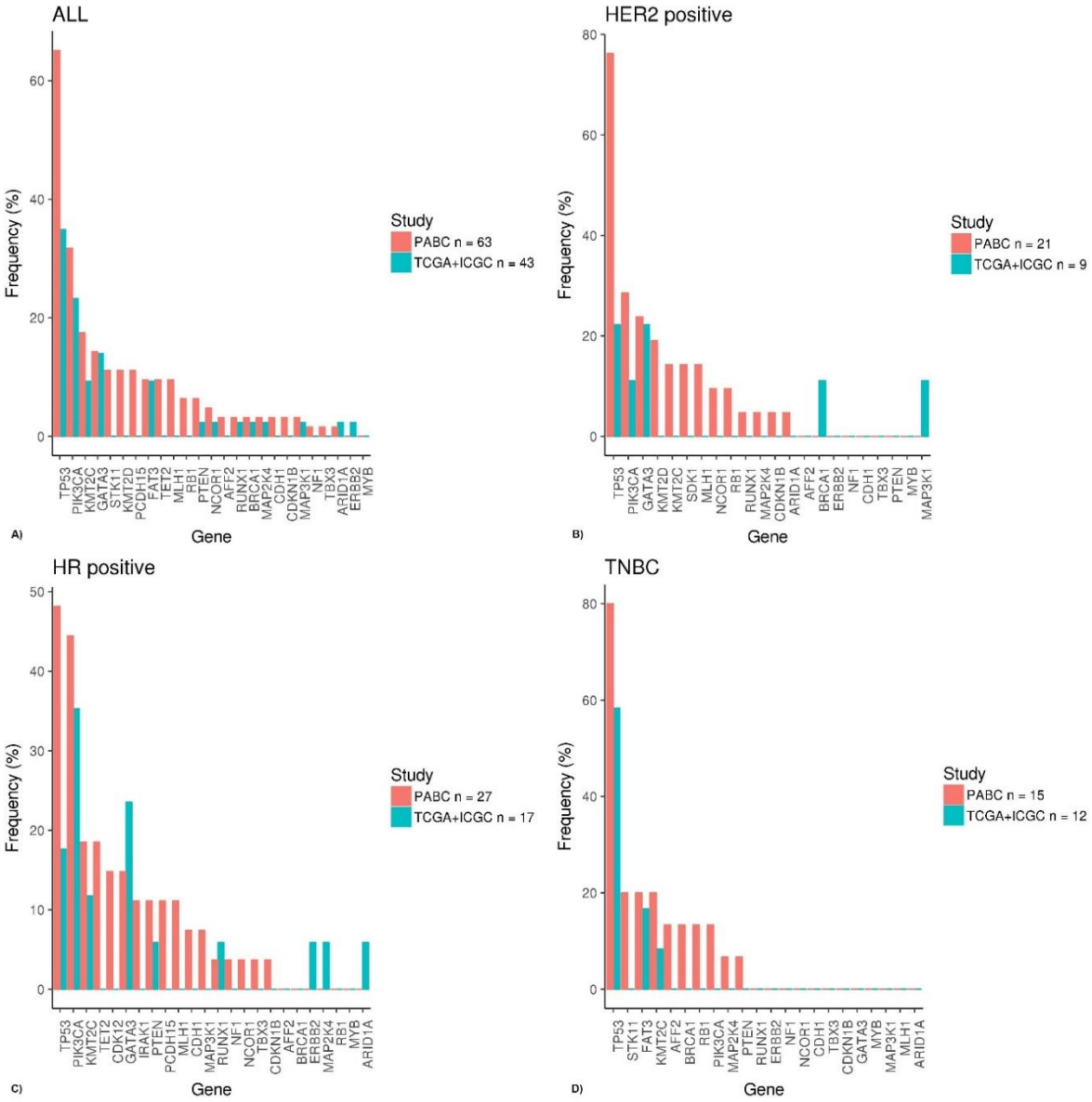

Figure 5.3: Mutation prevalence in our cohort (PABC: Premenopausal Asian Breast Cancers) and comparison to 43 premenopausal breast cancer patients who had relapsed from both TCGA and ICGC studies. (A) Comparison of all 63 samples in the cohort against 43 premenopausal breast cancer patients (all subtypes) who had relapsed from both TCGA and ICGC studies (25 non-Asian premenopausal from TCGA and 18 premenopausal from ICGC-EU). (B) Comparison of 21 HER2-positive samples against 9 TCGA + ICGC samples. (C) Comparison of 27 hormone receptor-positive samples against 17 TCGA + ICGC samples. (D) Comparison of 15 triple negative breast cancers against 12 TCGA + ICGC samples.

5.5 Discussion

While large scale sequencing projects have unraveled the complex architecture of breast cancers in the West [19-22], data on the genomic landscape of Asian breast cancers, including premenopausal tumours, remains limited [23,24]. Given that studies on next generation sequencing of Asian breast cancers that are currently published focus on special subsets, it is not possible to compare the mutation frequency from our cohort with an Asian premenopausal breast cancer cohort that has not relapsed. For example, in the study by Kim et al from Korea, whole exome sequencing was performed on 34 metastatic breast cancer specimens, with patient age ranging from 26.5 to 75.7 years [23]. In another study by Lee et al, also from Korea, a total of 78 normal-paired breast cancers were subjected to whole exome and RNA sequencing, but only 35 were from patients under the age of 50 years [24]. As illustrated in figures 2 and 3, the numbers of premenopausal non-Asian tumours profiled in large sequencing projects such as TCGA and ICGC are also limited. In this study, we have focused on characterizing the genomic profile of premenopausal Asian breast cancers which ultimately relapse, given that this is an area of unmet need. Similar to reports from other sequencing studies [19-21], there is a large number of genes where the frequency of alterations is <10%, reflecting the heterogeneity of the genomic landscape in breast cancer. Each tumour was also distinct with its own unique set of alterations.

The prevalence of *TP53* mutations is higher among the hormone receptor positive and HER2-positive subtypes in our cohort compared to non-Asian premenopausal cancers in TCGA, along with elevated frequencies of *TET2* and *CDK12* mutations among hormone receptor positive patients. The finding that *TP53* is more frequently mutated overall in our series may be related to the pre-selection of poor-prognosis tumours. However, this is also consistent with the findings from a recent study on unselected breast cancers from Chinese women. It reported no significant difference in the frequency of hormone receptor positive and HER2 negative tumours compared to the Caucasian series, but the prevalence of PAM50 luminal A subtype was lower and the prevalence of luminal B subtype was higher [25]. The frequency of HER2-overexpressing and basal-like breast cancers was similar between Chinese and Caucasian breast cancers. Luminal B subtypes are associated with more aggressive

biology and higher risk of relapse, and often harbour *TP53* mutations which have been implicated in resistance to endocrine and cytotoxic therapies [20,21,26,27]. In addition, the frequency of *TP53* mutations appears higher in our cohort compared to non-Asian premenopausal tumours in TCGA and ICGC that had relapsed, though this finding will require further validation in a larger study. These differences may possibly be related to differences in germline variants or polymorphisms, or environmental factors such as exposure to pollutants with oestrogenic effects [4]. While targeting the loss of function of tumour suppressor genes remains a major challenge, there may be opportunities in the near future with the advent of immune-based and epigenetic strategies, as well as compounds targeting downstream effectors in the affected pathways.

Although the presence of inactivating mutations in *NF1* was associated with inferior breast cancer-specific survival among ER negative tumours in the METABRIC study (hazard ratio 2.7; CI: 1.3-5.5) [28], with a different study reporting similar inferior outcomes more recently in ER positive tumours with *NF1* nonsense or frameshift mutations [29], only 1 of the 63 specimens in our series of poor prognosis was found to harbor mutation in *NF1* (2%). This is similar to frequencies reported in the large-scale sequencing projects of primary breast cancers which reported mutation frequency in *NF1* of 1% to 3.6% [28,30,31]. A study on the genomic evolution of breast cancer metastases also reported that *NF1* mutations were significantly enriched in the metastatic specimens [32], in keeping with the important role that *NF1* plays in breast cancer pathogenesis and progression. Our study focused on breast primary specimens, and amplification of *NF1*, with ploidy ranging from 2.1 to 3.4, was observed. The functional significance of gains in copy number of *NF1* is not known, though amplification of *NF1* has also been reported in breast cancers in other sequencing studies at frequencies of 1.6% to 2.5% [20,28] , with the frequency as high as 17% in 5 out of 29 breast cancer xenografts in another study [33] (http://www.cbioportal.org). Due to the nature of DNA from FFPE specimens, we could not reliably identify loss of *NF1* in the copy number analyses.

Other alterations present at higher frequencies in our series of poor-prognosis tumours include *CDK12* and *TET2*. CDK12 (cyclin-dependent kinase 12) is a regulatory kinase which protects cells from genomic instability. Recurrent *CDK12*

mutations in breast and ovarian cancers are associated with defects in DNA repair [34]. Silencing of *CDK12* has been shown to activate the mitogen-activated protein kinase (MAPK) signaling pathway, leading to endocrine therapy resistance through loss of ER dependence [35]. TET2 (tet-eleven translocation 2) from the TET family of DNA dioxygenases functions as DNA demethylases, antagonizing DNA methyltransferases-mediated DNA methylation and gene repression. Knockdown of *TET2* in breast cancer cells decreases epithelial cell adhesion molecule (EpCAM) and E-cadherin, increasing cell invasiveness [36].

MCL1 (myeloid cell leukaemia-1), a member of the anti-apoptotic pro-survival Bcl-2 family, can be amplified in all subtypes of breast cancer. In our cohort, *MCL1* amplification was predictive of inferior OS, likely related to the resistance to endocrine, cytotoxic and anti-HER2 therapies reported in preclinical studies [37,38]. The in vitro activity of MCL-1 inhibitors alone or in combination with other anti-cancer drugs provide a strong rationale for their clinical development [39,40].

Limitations of our study include the relatively small sample size, and the limited number of premenopausal breast cancers in TCGA and ICGC databases for comparison. Information on ethnicity was not documented for the ICGC cohort, although most of the patients were non-Asian. The suboptimal quality of DNA from older FFPE specimens also led to greater representation of more recent aggressive cases such as HER2-positive and triple negative cases which relapsed soon after initial diagnosis. This bias may partly explain why there were no significant survival differences among the different immunohistochemical subtypes, without association of younger age with worse survival. Conversely, increasing age was associated with worse OS in this selected premenopausal cohort, unlike studies which reported higher risk of relapse among younger women in non-metastatic cancers [7]. The retrospective nature of our study may also create some bias and heterogeneity, as it was not performed as a prospective cohort study or clinical trial. However, the data from this, and from similar studies[4,25], is sufficiently compelling to suggest that a detailed prospective study of Asian breast cancers by next generation sequencing is warranted.

While it is possible to identify increased copy number with the targeted gene panel, the low tumour purity of FFPE samples makes it difficult to distinguish between lack of tumour content and a true copy number loss. Hence the computational loss of heterozygosity calls in this cohort may not be reliable and are not reported. Germline DNA was not available for this study as a matched control. However our computational pipelines have been optimized to exclude germline and false positive sites, including for tumor suppressor genes. Lastly, we did not profile the recurrent specimens to interrogate the genomic evolution in the metastatic process. However, a recent study has demonstrated that new driver mutations private to the metastatic lesions are acquired later in the metastatic lineage [32]. Hence the primary tumour genome can still serve as a good proxy for the cells that seed the distant sites, and remains relevant in the development of therapeutic strategies in the adjuvant setting to prevent relapse.

5.6 Conclusions

In conclusion, our study has provided insights into the molecular profiles of Asian premenopausal breast cancer associated with relapse. The heterogeneity of breast cancers highlights the need to explore ethnic diversity in the genomic landscape. Standard systemic adjuvant therapies may be ineffective in these patients, and novel approaches exploiting the underlying tumour biology merit further research.

5.7 References

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5.8 Supplementary data

Supplementary Table S5.1: List of genes in NGDx PanCancer versión 2 panel

Gene	Gene.Name	Entrez_ID	Rationale
ABL1	c-abl oncogene 1, receptor tyrosine kinase	25	Known Cancer Gene
ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)	27	Copy Number Gene
ACTL6A	actin-like 6A	86	Exploratory Gene
ACTL6B	actin-like 6B	51412	Exploratory Gene
ACVR1B	activin A receptor, type IB	91	Known Cancer Gene
ACVR2A	activin A receptor type 2A	92	Exploratory Gene
AFF2	AF4/FMR2 family, member 2	2334	Known Cancer Gene
AKT1	v-akt murine thymoma viral oncogene homolog 1	207	Known Cancer Gene
AKT2	v-akt murine thymoma viral oncogene homolog 2	208	Known Cancer Gene
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	10000	Known Cancer Gene
ALK	anaplastic lymphoma receptor tyrosine kinase	238	Known Cancer Gene
ALOX12B	arachidonate 12-lipoxygenase, 12R type	242	Exploratory Gene
AMER1	APC membrane recruitment protein 1	139285	Exploratory Gene
ANAPC1	anaphase promoting complex subunit 1	64682	Exploratory Gene
ANO1	anoctamin 1, calcium activated chloride channel	55107	Exploratory Gene
APC	adenomatous polyposis coli	324	Known Cancer Gene
AR	androgen receptor	367	Copy Number Gene
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	369	Exploratory Gene
ARFRP1	ADP-ribosylation factor related protein 1	10139	Exploratory Gene
ARHGEF39		84904	Known Cancer Gene
ARID1A	AT rich interactive domain 1A (SWI-like)	8289	Known Cancer Gene
ARID1B	AT rich interactive domain 1B (SWI1-like)	57492	Exploratory Gene
ARID2	AT rich interactive domain 2 (ARID, RFX-like)	196528	Known Cancer Gene
ARID5B	AT-rich interaction domain 5B	84159	Exploratory Gene
ATAD2	ATPase family, AAA domain containing 2	29028	Exploratory Gene
ATM	similar to Serine-protein kinase ATM (Ataxia telangiectasia mutated) (A-T, mutated); ataxia telangiectasia mutated	651610	Known Cancer Gene

ATR	ataxia telangiectasia and Rad3 related; similar to ataxia telangiectasia and Rad3 related protein	651921	Exploratory Gene
ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	546	Known Cancer Gene
AURKA	aurora kinase A; aurora kinase A pseudogene 1	6791	Exploratory Gene
AURKB	aurora kinase B	9212	Exploratory Gene
AXIN1	axin 1	8312	Known Cancer Gene
AXL	AXL receptor tyrosine kinase	558	Known Cancer Gene
B3GAT1	beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	27087	Copy Number Gene
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	571	Known Cancer Gene
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	60468	Copy Number Gene
BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	8314	Known Cancer Gene
BARD1	BRCA1 associated RING domain 1	580	Known Cancer Gene
BCL11A	B-cell CLL/lymphoma 11A	53335	Exploratory Gene
BCL11B	B-cell CLL/lymphoma 11B	64919	Exploratory Gene
BCL2A1	BCL2-related protein A1	597	Exploratory Gene
BCL2L1	BCL2-like 1	598	Copy Number Gene
BCL2L11	BCL2-like 11 (apoptosis facilitator)	10018	Known Cancer Gene
BCL3	B-cell CLL/lymphoma 3	602	Copy Number Gene
BCL6	B-cell CLL/lymphoma 6	604	Exploratory Gene
BCL7A	BCL tumor suppressor 7A	605	Exploratory Gene
BCL7B	BCL tumor suppressor 7B	9275	Exploratory Gene
BCL7C	BCL tumor suppressor 7C	9274	Exploratory Gene
BCOR	BCL6 co-repressor	54880	Known Cancer Gene
BDH1	3-hydroxybutyrate dehydrogenase, type 1	622	Known Cancer Gene
BIRC2	baculoviral IAP repeat-containing 2	329	Copy Number Gene
BIRC3	baculoviral IAP repeat-containing 3	330	Exploratory Gene
BIRC7	baculoviral IAP repeat-containing 7	79444	Copy Number Gene
BLM	Bloom syndrome, RecQ helicase-like	641	Known Cancer Gene
BOK	BCL2-related ovarian killer	666	Copy Number Gene
BRAF	v-raf murine sarcoma viral oncogene homolog B1	673	Known Cancer Gene
BRCA1	breast cancer 1, early onset	672	Known Cancer Gene
BRCA2	breast cancer 2, early onset	675	Known Cancer Gene
BRD4	bromodomain containing 4	23476	Exploratory Gene
BRD7	bromodomain containing 7; bromodomain containing 7 pseudogene 2	29117	Exploratory Gene

BRD9	bromodomain containing 9	65980	Exploratory Gene
BRIP1	BRCA1 interacting protein C-terminal helicase 1	83990	Exploratory Gene
BTG1	B-cell translocation gene 1, anti-proliferative	694	Exploratory Gene
BTK	Bruton agammaglobulinemia tyrosine kinase	695	Exploratory Gene
C11orf30	chromosome 11 open reading frame 30	56946	Exploratory Gene
CABLES1	Cdk5 and Abl enzyme substrate 1	91768	Known Cancer Gene
CARD11	caspase recruitment domain family, member 11	84433	Exploratory Gene
CASP8	caspase 8, apoptosis-related cysteine peptidase	841	Exploratory Gene
CBFB	core-binding factor, beta subunit	865	Known Cancer Gene
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	867	Known Cancer Gene
CCND1	cyclin D1	595	Copy Number Gene
CCND2	cyclin D2	894	Exploratory Gene
CCND3	cyclin D3	896	Exploratory Gene
CCNE1	cyclin E1	898	Copy Number Gene
CCSER1	coiled-coil serine rich protein 1	401145	Exploratory Gene
CD19	CD19 molecule	930	Exploratory Gene
CD274	CD274 molecule	29126	Exploratory Gene
CD79A	CD79a molecule, immunoglobulin-associated alpha	973	Exploratory Gene
CD79B	CD79b molecule, immunoglobulin-associated beta	974	Exploratory Gene
CDA	cytidine deaminase	978	Known Cancer Gene
CDC73	cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	79577	Exploratory Gene
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	999	Known Cancer Gene
CDH13	cadherin 13, H-cadherin (heart)	1012	Copy Number Gene
CDH20	cadherin 20, type 2	28316	Copy Number Gene
CDH3	cadherin 3	1001	Exploratory Gene
CDH5	cadherin 5, type 2 (vascular endothelium)	1003	Copy Number Gene
CDH6	cadherin 6	1004	Exploratory Gene
CDK12	Cdc2-related kinase, arginine/serine-rich	51755	Exploratory Gene
CDK17	PCTAIRE protein kinase 2	5128	Known Cancer Gene
CDK2	cyclin dependent kinase 2	1017	Exploratory Gene
CDK4	cyclin-dependent kinase 4	1019	Exploratory Gene
CDK6	cyclin-dependent kinase 6	1021	Exploratory Gene
CDKN1A	cyclin dependent kinase inhibitor 1A	1026	Exploratory Gene

CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1027	Known Cancer Gene
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	1029	Known Cancer Gene
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1030	Copy Number Gene
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	1031	Exploratory Gene
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	1050	Exploratory Gene
CHD1	chromodomain helicase DNA binding protein 1	1105	Known Cancer Gene
CHD3	chromodomain helicase DNA binding protein 3	1107	Known Cancer Gene
CHD4	chromodomain helicase DNA binding protein 4	1108	Known Cancer Gene
CHD5	chromodomain helicase DNA binding protein 5	26038	Exploratory Gene
CHEK1	CHK1 checkpoint homolog (S. pombe)	1111	Known Cancer Gene
CHEK2	protein kinase CHK2-like; CHK2 checkpoint homolog (S. pombe); similar to hCG1983233	11200	Exploratory Gene
CHFR	checkpoint with forkhead and ring finger domains	55743	Copy Number Gene
CHSY3	chondroitin sulfate synthase 3	337876	Known Cancer Gene
CHUK	conserved helix-loop-helix ubiquitous kinase	1147	Exploratory Gene
CIC	capicua homolog (Drosophila)	23152	Known Cancer Gene
COPS5	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	10987	Exploratory Gene
COX18	COX18 cytochrome c oxidase assembly homolog (S. cerevisiae)	285521	Known Cancer Gene
CPSF3	cleavage and polyadenylation specific factor 3	51692	Exploratory Gene
CREBBP	CREB binding protein	1387	Known Cancer Gene
CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	1399	Copy Number Gene
CSF1	colony stimulating factor 1	1435	Exploratory Gene
CSF1R	colony stimulating factor 1 receptor	1436	Exploratory Gene
CTCF	CCCTC-binding factor (zinc finger protein)	10664	Known Cancer Gene
CTLA4	cytotoxic T-lymphocyte-associated protein 4	1493	Copy Number Gene
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	1499	Known Cancer Gene
CUL1	cullin 1	8454	Known Cancer Gene

CUL4A	cullin 4A	8451	Exploratory Gene
CUL4B	cullin 4B	8450	Exploratory Gene
CUX1	cut like homeobox 1	1523	Exploratory Gene
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	1586	Known Cancer Gene
DAXX	death-domain associated protein	1616	Known Cancer Gene
DCC	deleted in colorectal carcinoma	1630	Known Cancer Gene
DDR2	discoidin domain receptor tyrosine kinase 2	4921	Known Cancer Gene
DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)	1663	Copy Number Gene
DEPDC5	DEP domain containing 5	9681	Exploratory Gene
DICER1	dicer 1, ribonuclease type III	23405	Known Cancer Gene
DIS3	DIS3 mitotic control homolog (S. cerevisiae)	22894	Exploratory Gene
DKK1	dickkopf homolog 1 (Xenopus laevis)	22943	Exploratory Gene
DLGAP2	discs, large (Drosophila) homolog-associated protein 2	9228	Copy Number Gene
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	1788	Known Cancer Gene
DOT1L	DOT1-like, histone H3 methyltransferase (S. cerevisiae)	84444	Exploratory Gene
DPF1	D4, zinc and double PHD fingers family 1	8193	Exploratory Gene
DPF2	D4, zinc and double PHD fingers family 2	5977	Exploratory Gene
DPF3	D4, zinc and double PHD fingers, family 3	8110	Exploratory Gene
DSG3	desmoglein 3 (pemphigus vulgaris antigen)	1830	Known Cancer Gene
DTX2	deltex homolog 2 (Drosophila)	113878	Copy Number Gene
DUSP4	dual specificity phosphatase 4	1846	Copy Number Gene
EED	embryonic ectoderm development	8726	Exploratory Gene
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1956	Known Cancer Gene
EIF1AX	eukaryotic translation initiation factor 1A, X-linked	1964	Exploratory Gene
ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)	2004	Copy Number Gene
ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)	2005	Known Cancer Gene
ELMO1	engulfment and cell motility 1	9844	Exploratory Gene
EML4	echinoderm microtubule associated protein like 4	27436	Known Cancer Gene
EP300	E1A binding protein p300	2033	Known Cancer Gene
EPAS1	endothelial PAS domain protein 1	2034	Exploratory Gene

EPHA2	EPH receptor A2	1969	Known Cancer Gene
EPHA3	EPH receptor A3	2042	Known Cancer Gene
EPHA6	EPH receptor A6	285220	Known Cancer Gene
EPHA7	EPH receptor A7	2045	Known Cancer Gene
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	2064	Known Cancer Gene
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	2065	Exploratory Gene
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	2066	Known Cancer Gene
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)	2067	Known Cancer Gene
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2	2068	Known Cancer Gene
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)	2078	Known Cancer Gene
ESR1	estrogen receptor 1	2099	Exploratory Gene
ESR2	estrogen receptor 2 (ER beta)	2100	Exploratory Gene
ETV1	ets variant 1	2115	Known Cancer Gene
ETV4	ets variant 4	2118	Known Cancer Gene
ETV5	ets variant 5	2119	Known Cancer Gene
ETV6	ets variant 6	2120	Known Cancer Gene
EWSR1	similar to Ewing sarcoma breakpoint region 1; Ewing sarcoma breakpoint region 1	2130	Known Cancer Gene
EZH2	enhancer of zeste homolog 2 (Drosophila)	2146	Known Cancer Gene
FANCA	Fanconi anemia, complementation group A	2175	Exploratory Gene
FANCC	Fanconi anemia, complementation group C	2176	Exploratory Gene
FANCD2	Fanconi anemia, complementation group D2	2177	Exploratory Gene
FANCE	Fanconi anemia, complementation group E	2178	Exploratory Gene
FANCG	Fanconi anemia, complementation group G	2189	Exploratory Gene
FANCI	Fanconi anemia, complementation group I	55215	Exploratory Gene
FANCL	Fanconi anemia, complementation group L	55120	Exploratory Gene

FANCM	Fanconi anemia, complementation group M	57697	Exploratory Gene
FAT1	FAT tumor suppressor homolog 1 (Drosophila)	2195	Known Cancer Gene
FAT3	FAT tumor suppressor homolog 3 (Drosophila)	120114	Exploratory Gene
FBXW7	F-box and WD repeat domain containing 7	55294	Known Cancer Gene
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	2212	Known Cancer Gene
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	2214	Known Cancer Gene
FGF10	fibroblast growth factor 10	2255	Exploratory Gene
FGF12	fibroblast growth factor 12	2257	Exploratory Gene
FGF14	fibroblast growth factor 14	2259	Exploratory Gene
FGF19	fibroblast growth factor 19	9965	Exploratory Gene
FGF23	fibroblast growth factor 23	8074	Exploratory Gene
FGF3	fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homolog)	2248	Exploratory Gene
FGF4	fibroblast growth factor 4	2249	Exploratory Gene
FGF6	fibroblast growth factor 6	2251	Exploratory Gene
FGFR1	fibroblast growth factor receptor 1	2260	Known Cancer Gene
FGFR2	fibroblast growth factor receptor 2	2263	Known Cancer Gene
FGFR3	fibroblast growth factor receptor 3	2261	Known Cancer Gene
FGFR4	fibroblast growth factor receptor 4	2264	Known Cancer Gene
FH	fumarate hydratase	2271	Copy Number Gene
FLG	filaggrin	2312	Copy Number Gene
FLG2	filaggrin family member 2	388698	Known Cancer Gene
FLI1	Friend leukemia virus integration 1	2313	Known Cancer Gene
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	2321	Known Cancer Gene
FLT3	fms-related tyrosine kinase 3	2322	Known Cancer Gene
FLT4	fms-related tyrosine kinase 4	2324	Copy Number Gene
FOXA1	forkhead box A1	3169	Known Cancer Gene
FOXC1	forkhead box C1	2296	Copy Number Gene
FOXL2	forkhead box L2	668	Known Cancer Gene
FOXM1	forkhead box M1	2305	Copy Number Gene
FOXP1	forkhead box P1	27086	Exploratory Gene
FRK	fyn related Src family tyrosine kinase	2444	Exploratory Gene
FRS2	fibroblast growth factor receptor substrate 2	10818	Known Cancer Gene
GAB2	GRB2-associated binding protein 2	9846	Copy Number Gene
GATA1	GATA binding protein 1 (globin transcription factor 1)	2623	Known Cancer Gene

GATA3	GATA binding protein 3	2625	Known Cancer Gene
GLI1		2735	Exploratory Gene
GMDS	GDP-mannose 4,6-dehydratase	2762	Known Cancer Gene
GNA11	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	2767	Known Cancer Gene
GNAQ	guanine nucleotide binding protein (G protein), q polypeptide	2776	Known Cancer Gene
GNAS	GNAS complex locus	2778	Known Cancer Gene
GPC6	glypican 6	10082	Copy Number Gene
GPR124	G protein-coupled receptor 124	25960	Exploratory Gene
GPS2	G protein pathway suppressor 2	2874	Known Cancer Gene
GRB2	growth factor receptor-bound protein 2	2885	Copy Number Gene
GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A	2903	Exploratory Gene
GSK3B	glycogen synthase kinase 3 beta	2932	Copy Number Gene
H3F3A	H3 histone, family 3B (H3.3B); H3 histone, family 3A pseudogene; H3 histone, family 3A; similar to H3 histone, family 3B; similar to histone H3.3B	3021	Known Cancer Gene
HAVCR2	hepatitis A virus cellular receptor 2	84868	Exploratory Gene
HAX1	HCLS1 associated protein X-1	10456	Known Cancer Gene
HDAC10	histone deacetylase 10	83933	Known Cancer Gene
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	3082	Exploratory Gene
HIF1A	hypoxia inducible factor 1 alpha subunit	3091	Exploratory Gene
HLA-A	major histocompatibility complex, class I, A	3105	Exploratory Gene
HLA-B	major histocompatibility complex, class I, B	3106	Exploratory Gene
HLA-C	major histocompatibility complex, class I, C	3107	Exploratory Gene
HNF4A	hepatocyte nuclear factor 4 alpha	3172	Exploratory Gene
HOXA13	homeobox A13	3209	Known Cancer Gene
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	3265	Known Cancer Gene
HRNR	hornerin	388697	Known Cancer Gene
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 2; heat shock protein 90kDa alpha (cytosolic), class A member 1	3320	Exploratory Gene
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	3326	Exploratory Gene
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	7184	Exploratory Gene
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	3417	Known Cancer Gene

IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	3418	Known Cancer Gene
IFNG	interferon, gamma	3458	Known Cancer Gene
IGF1	insulin-like growth factor 1 (somatomedin C)	3479	Exploratory Gene
IGF1R	insulin-like growth factor 1 receptor	3480	Copy Number Gene
IGF2	insulin-like growth factor 2 (somatomedin A); insulin; INS-IGF2 readthrough transcript	3481	Copy Number Gene
IGF2R	insulin-like growth factor 2 receptor	3482	Exploratory Gene
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	9641	Copy Number Gene
IL7R	interleukin 7 receptor	3575	Known Cancer Gene
ING1	inhibitor of growth family, member 1	3621	Known Cancer Gene
INPP4B	inositol polyphosphate-4-phosphatase type II B	8821	Exploratory Gene
INSR	insulin receptor	3643	Exploratory Gene
INTS4	integrator complex subunit 4	92105	Known Cancer Gene
IRAK1	interleukin-1 receptor-associated kinase 1	3654	Copy Number Gene
IRF2	interferon regulatory factor 2	3660	Exploratory Gene
IRF4	interferon regulatory factor 4	3662	Exploratory Gene
IRS2	insulin receptor substrate 2	8660	Copy Number Gene
JAK1	Janus kinase 1	3716	Exploratory Gene
JAK2	Janus kinase 2	3717	Exploratory Gene
JAK3	Janus kinase 3	3718	Known Cancer Gene
JUB	jub, ajuba homolog (Xenopus laevis)	84962	Known Cancer Gene
KAT6A		7994	Exploratory Gene
KAT6B		23522	Copy Number Gene
KDM1A	lysine (K)-specific demethylase 1	23028	Exploratory Gene
KDM2A	lysine (K)-specific demethylase 2A	22992	Exploratory Gene
KDM2B	lysine (K)-specific demethylase 2B	84678	Exploratory Gene
KDM3B	lysine (K)-specific demethylase 3B	51780	Exploratory Gene
KDM4A	lysine (K)-specific demethylase 4A	9682	Exploratory Gene
KDM4B	lysine (K)-specific demethylase 4B	23030	Exploratory Gene
KDM4C	lysine (K)-specific demethylase 4C	23081	Exploratory Gene
KDM5A	lysine (K)-specific demethylase 5A	5927	Known Cancer Gene
KDM5C	lysine (K)-specific demethylase 5C	8242	Known Cancer Gene
KDM6A	lysine (K)-specific demethylase 6A	7403	Known Cancer Gene
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	3791	Known Cancer Gene
KEAP1	kelch-like ECH-associated protein 1	9817	Known Cancer Gene
KIF5B	kinesin family member 5B	3799	Known Cancer Gene
KIT	similar to Mast/stem cell growth factor receptor precursor(SCFR) (Proto-oncogene tyrosine-protein kinase Kit)	652799	Known Cancer Gene

	(c-kit) (CD117 antigen); v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog		
KLF4	Kruppel-like factor 4 (gut)	9314	Exploratory Gene
KLHL6	kelch-like 6 (Drosophila)	89857	Exploratory Gene
KMT2A	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	4297	Known Cancer Gene
KMT2B	myeloid/lymphoid or mixed-lineage leukemia 4	9757	Known Cancer Gene
KMT2C	myeloid/lymphoid or mixed-lineage leukemia 3	58508	Known Cancer Gene
KMT2D	myeloid/lymphoid or mixed-lineage leukemia 2	8085	Known Cancer Gene
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	3845	Known Cancer Gene
KYNU	kynureninase (L-kynurenine hydrolase)	8942	Copy Number Gene
LAG3	lymphocyte activating 3	3902	Exploratory Gene
LATS1	LATS, large tumor suppressor, homolog 1 (Drosophila)	9113	Exploratory Gene
LATS2	LATS, large tumor suppressor, homolog 2 (Drosophila)	26524	Exploratory Gene
LMO1	LIM domain only 1 (rhombotin 1)	4004	Known Cancer Gene
LONRF1	LON peptidase N-terminal domain and ring finger 1	91694	Known Cancer Gene
LTK	leukocyte receptor tyrosine kinase	4058	Known Cancer Gene
MACROD2	MACRO domain containing 2	140733	Known Cancer Gene
MAP2K1	mitogen-activated protein kinase kinase 1	5604	Known Cancer Gene
MAP2K2	mitogen-activated protein kinase kinase 2 pseudogene; mitogen-activated protein kinase kinase 2	5605	Known Cancer Gene
MAP2K4	mitogen-activated protein kinase kinase 4	6416	Known Cancer Gene
MAP3K1	mitogen-activated protein kinase kinase kinase 1	4214	Known Cancer Gene
MAP3K13	mitogen-activated protein kinase kinase kinase 13	9175	Known Cancer Gene
MAP3K7	mitogen-activated protein kinase kinase kinase 7	6885	Known Cancer Gene
MAPK1	mitogen-activated protein kinase 1	5594	Exploratory Gene
MAPK3	hypothetical LOC100271831; mitogen-activated protein kinase 3	5595	Exploratory Gene
MAPK7	mitogen-activated protein kinase 7	5598	Copy Number Gene
MAPKBP1	mitogen-activated protein kinase binding protein 1	23005	Known Cancer Gene

MAX	MYC associated factor X	4149	Copy Number Gene
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	4170	Copy Number Gene
MDM2	Mdm2 p53 binding protein homolog (mouse)	4193	Copy Number Gene
MDM4	Mdm4 p53 binding protein homolog (mouse)	4194	Copy Number Gene
MECOM	ecotropic viral integration site 1	2122	Copy Number Gene
MED12	mediator complex subunit 12	9968	Known Cancer Gene
MELK	maternal embryonic leucine zipper kinase	9833	Copy Number Gene
MEN1	multiple endocrine neoplasia I	4221	Known Cancer Gene
MET	met proto-oncogene (hepatocyte growth factor receptor)	4233	Known Cancer Gene
MGMT	O-6-methylguanine-DNA methyltransferase	4255	Copy Number Gene
MITF	microphthalmia-associated transcription factor	4286	Copy Number Gene
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	4292	Known Cancer Gene
MLH3	mutL homolog 3 (E. coli)	27030	Known Cancer Gene
MOB1A		55233	Exploratory Gene
MOB1B		92597	Exploratory Gene
MORC1	MORC family CW-type zinc finger 1	27136	Known Cancer Gene
MPL	myeloproliferative leukemia virus oncogene	4352	Known Cancer Gene
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	4436	Known Cancer Gene
MSH3	mutS homolog 3 (E. coli)	4437	Known Cancer Gene
MSH6	mutS homolog 6 (E. coli)	2956	Known Cancer Gene
MST1	macrophage stimulating 1 (hepatocyte growth factor-like)	4485	Exploratory Gene
MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	4486	Exploratory Gene
MTAP	methylthioadenosine phosphorylase	4507	Exploratory Gene
MTOR	mechanistic target of rapamycin (serine/threonine kinase)	2475	Exploratory Gene
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	4602	Copy Number Gene
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	4609	Copy Number Gene
MYCL	MYCL proto-oncogene, bHLH transcription factor	4610	Exploratory Gene
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	4613	Copy Number Gene

MYH9	myosin, heavy chain 9, non-muscle	4627	Known Cancer Gene
NAALADL2	N-acetylated alpha-linked acidic dipeptidase-like 2	254827	Copy Number Gene
NCOR1	nuclear receptor co-repressor 1	9611	Known Cancer Gene
NEGR1	neuronal growth regulator 1	257194	Copy Number Gene
NF1	neurofibromin 1	4763	Known Cancer Gene
NF2	neurofibromin 2 (merlin)	4771	Known Cancer Gene
NFE2L2	nuclear factor (erythroid-derived 2)-like 2	4780	Known Cancer Gene
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4792	Copy Number Gene
NKX2-1	NK2 homeobox 1	7080	Copy Number Gene
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	4851	Known Cancer Gene
NOTCH2	Notch homolog 2 (Drosophila)	4853	Known Cancer Gene
NOTCH3	Notch homolog 3 (Drosophila)	4854	Copy Number Gene
NOTCH4	Notch homolog 4 (Drosophila)	4855	Known Cancer Gene
NPM1	nucleophosmin 1 (nucleolar phosphoprotein B23, numatrin) pseudogene 21; hypothetical LOC100131044; similar to nucleophosmin 1; nucleophosmin (nucleolar phosphoprotein B23, numatrin)	729686	Known Cancer Gene
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	4893	Known Cancer Gene
NSD1	nuclear receptor binding SET domain protein 1	64324	Known Cancer Gene
NT5C2	5'-nucleotidase, cytosolic II	22978	Known Cancer Gene
NTNG1	netrin G1	22854	Known Cancer Gene
NTRK1	neurotrophic tyrosine kinase, receptor, type 1	4914	Exploratory Gene
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	4915	Exploratory Gene
NTRK3	neurotrophic tyrosine kinase, receptor, type 3	4916	Known Cancer Gene
PAF1	Paf1, RNA polymerase II associated factor, homolog (S. cerevisiae)	54623	Known Cancer Gene
PAK7	p21 protein (Cdc42/Rac)-activated kinase 7	57144	Known Cancer Gene
PALB2	partner and localizer of BRCA2	79728	Exploratory Gene
PARD6G	par-6 partitioning defective 6 homolog gamma (C. elegans)	84552	Copy Number Gene
PARK2	Parkinson disease (autosomal recessive, juvenile) 2, parkin	5071	Copy Number Gene

PARP1	poly (ADP-ribose) polymerase 1	142	Known Cancer Gene
PARP10	poly (ADP-ribose) polymerase family, member 10	84875	Known Cancer Gene
PARP2	poly (ADP-ribose) polymerase 2	10038	Known Cancer Gene
PARP3	poly (ADP-ribose) polymerase family, member 3	10039	Known Cancer Gene
PARP4	poly (ADP-ribose) polymerase family, member 4	143	Known Cancer Gene
PAX3	paired box 3	5077	Exploratory Gene
PAX8	paired box 8	7849	Known Cancer Gene
PBRM1	polybromo 1	55193	Known Cancer Gene
PCDH15	protocadherin 15	65217	Known Cancer Gene
PDCD1	programmed cell death 1	5133	Exploratory Gene
PDCD1LG2	programmed cell death 1 ligand 2	80380	Exploratory Gene
PDE4D	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	5144	Known Cancer Gene
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	5156	Known Cancer Gene
PDGFRB	platelet-derived growth factor receptor, beta polypeptide	5159	Known Cancer Gene
PDK1	pyruvate dehydrogenase kinase, isozyme 1	5163	Exploratory Gene
PDPK1	3-phosphoinositide dependent protein kinase-1	5170	Exploratory Gene
PEA15	phosphoprotein enriched in astrocytes 15	8682	Copy Number Gene
PEG10	paternally expressed 10	23089	Known Cancer Gene
PGM5	phosphoglucomutase 5	5239	Exploratory Gene
PHF10	PHD finger protein 10	55274	Exploratory Gene
PHF12	PHD finger protein 12	57649	Known Cancer Gene
PHF6	PHD finger protein 6	84295	Known Cancer Gene
PHLPP2	PH domain and leucine rich repeat protein phosphatase 2	23035	Exploratory Gene
PHOX2B	paired-like homeobox 2b	8929	Known Cancer Gene
PIK3C2G	phosphoinositide-3-kinase, class 2, gamma polypeptide	5288	Known Cancer Gene
PIK3C3	phosphoinositide-3-kinase, class 3	5289	Exploratory Gene
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	5290	Known Cancer Gene
PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide	5291	Exploratory Gene
PIK3CG	phosphoinositide-3-kinase, catalytic, gamma polypeptide	5294	Exploratory Gene
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	5295	Known Cancer Gene

PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2 (beta)	5296	Known Cancer Gene
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	8503	Exploratory Gene
PIK3R4	phosphoinositide-3-kinase, regulatory subunit 4	30849	Exploratory Gene
PIK3R5	phosphoinositide-3-kinase, regulatory subunit 5	23533	Exploratory Gene
PIK3R6	phosphoinositide-3-kinase, regulatory subunit 6	146850	Exploratory Gene
PIM1	pim-1 oncogene	5292	Exploratory Gene
PIM2	pim-2 oncogene	11040	Exploratory Gene
PIM3	pim-3 oncogene	415116	Exploratory Gene
PKHD1	polycystic kidney and hepatic disease 1 (autosomal recessive)	5314	Exploratory Gene
PLAT	plasminogen activator, tissue	5327	Copy Number Gene
PLK2	polo-like kinase 2 (Drosophila)	10769	Copy Number Gene
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)	5395	Exploratory Gene
POLD1	polymerase (DNA directed), delta 1, catalytic subunit 125kDa	5424	Exploratory Gene
POLE	polymerase (DNA directed), epsilon	5426	Known Cancer Gene
PORCN	porcupine homolog (Drosophila)	64840	Exploratory Gene
PPARA	peroxisome proliferator-activated receptor alpha	5465	Copy Number Gene
PPARG	peroxisome proliferator-activated receptor gamma	5468	Known Cancer Gene
PPM1E	protein phosphatase 1E (PP2C domain containing)	22843	Known Cancer Gene
PPP1R3A	protein phosphatase 1, regulatory (inhibitor) subunit 3A	5506	Known Cancer Gene
PPP2R1A	protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	5518	Known Cancer Gene
PPP6C	protein phosphatase 6, catalytic subunit	5537	Exploratory Gene
PRDM1	PR domain containing 1, with ZNF domain	639	Known Cancer Gene
PREX2	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	80243	Known Cancer Gene
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	5573	Exploratory Gene
PRKCA	protein kinase C, alpha	5578	Exploratory Gene
PRKCB	protein kinase C, beta	5579	Exploratory Gene
PRKCG	protein kinase C, gamma	5582	Exploratory Gene

PRKDC	similar to protein kinase, DNA-activated, catalytic polypeptide; protein kinase, DNA-activated, catalytic polypeptide	731751	Exploratory Gene
PRLR	prolactin receptor	5618	Exploratory Gene
PRMT5	protein arginine methyltransferase 5	10419	Exploratory Gene
PROZ	protein Z, vitamin K-dependent plasma glycoprotein	8858	Copy Number Gene
PRRX1	paired related homeobox 1	5396	Known Cancer Gene
PRSS1	protease, serine, 1 (trypsin 1); trypsinogen C	154754	Copy Number Gene
PRX	periaxin	57716	Known Cancer Gene
PTCH1	patched homolog 1 (Drosophila)	5727	Known Cancer Gene
PTCH2	patched homolog 2 (Drosophila)	8643	Exploratory Gene
PTEN	phosphatase and tensin homolog; phosphatase and tensin homolog pseudogene 1	5728	Known Cancer Gene
PTP4A1	protein tyrosine phosphatase type IVA, member 1	7803	Copy Number Gene
PTPN11	protein tyrosine phosphatase, non-receptor type 11; similar to protein tyrosine phosphatase, non-receptor type 11	442113	Known Cancer Gene
PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	26191	Known Cancer Gene
PTPRD	protein tyrosine phosphatase, receptor type, D	5789	Copy Number Gene
PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	5799	Known Cancer Gene
RABGEF1	RAB guanine nucleotide exchange factor (GEF) 1	27342	Copy Number Gene
RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	5879	Known Cancer Gene
RAD21	RAD21 homolog (S. pombe)	5885	Copy Number Gene
RAD50	RAD50 homolog (S. cerevisiae)	10111	Exploratory Gene
RAD51	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	5888	Exploratory Gene
RAD51B		5890	Exploratory Gene
RAD51C	RAD51 homolog C (S. cerevisiae)	5889	Exploratory Gene
RAD51D		5892	Exploratory Gene
RAD52	RAD52 homolog (S. cerevisiae)	5893	Exploratory Gene
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	5894	Known Cancer Gene
RALA	RAS like proto-oncogene A	5898	Exploratory Gene
RALB	RAS like proto-oncogene B	5899	Exploratory Gene

RB1	retinoblastoma 1	5925	Known Cancer Gene
RBFOX1		54715	Copy Number Gene
RBM10	RNA binding motif protein 10	8241	Known Cancer Gene
REG4	regenerating islet-derived family, member 4	83998	Copy Number Gene
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	5966	Exploratory Gene
RET	ret proto-oncogene	5979	Known Cancer Gene
RHEB	Ras homolog enriched in brain	6009	Known Cancer Gene
RHOA	ras homolog family member A	387	Exploratory Gene
RICTOR	RPTOR independent companion of MTOR, complex 2	253260	Exploratory Gene
RIOK3	RIO kinase 3 (yeast)	8780	Copy Number Gene
RIT1	Ras like without CAAX 1	6016	Exploratory Gene
RNF19A	ring finger protein 19A	25897	Copy Number Gene
RNF32	ring finger protein 32	140545	Copy Number Gene
RNF43	ring finger protein 43	54894	Known Cancer Gene
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila); similar to roundabout 1 isoform b	642132	Known Cancer Gene
ROBO2	roundabout, axon guidance receptor, homolog 2 (Drosophila)	6092	Known Cancer Gene
ROS1	c-ros oncogene 1 , receptor tyrosine kinase	6098	Known Cancer Gene
RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5	9252	Copy Number Gene
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	6198	Copy Number Gene
RPS6KC1	ribosomal protein S6 kinase, 52kDa, polypeptide 1	26750	Known Cancer Gene
RPTN	repetin	126638	Known Cancer Gene
RPTOR	regulatory associated protein of MTOR, complex 1	57521	Exploratory Gene
RSPO1	R-spondin homolog (Xenopus laevis)	284654	Known Cancer Gene
RSPO2	R-spondin 2 homolog (Xenopus laevis)	340419	Known Cancer Gene
RSPO3	R-spondin 3 homolog (Xenopus laevis)	84870	Known Cancer Gene
RSPO4	R-spondin family, member 4	343637	Exploratory Gene
RUNX1	runt-related transcription factor 1	861	Known Cancer Gene
SAV1	salvador homolog 1 (Drosophila)	60485	Exploratory Gene
SDK1	sidekick homolog 1, cell adhesion molecule (chicken); hypothetical LOC730351	730351	Known Cancer Gene
SELL	selectin L	6402	Copy Number Gene
SETD2	SET domain containing 2	29072	Known Cancer Gene
SF3B1	splicing factor 3b, subunit 1, 155kDa	23451	Known Cancer Gene
SFRP1	secreted frizzled-related protein 1	6422	Copy Number Gene

SGK1	serum/glucocorticoid regulated kinase 1	6446	Exploratory Gene
SH2B3	SH2B adaptor protein 3	10019	Exploratory Gene
SIM1	single-minded homolog 1 (Drosophila)	6492	Copy Number Gene
SKP2	S-phase kinase associated protein 2	6502	Exploratory Gene
SLIT2	slit homolog 2 (Drosophila)	9353	Known Cancer Gene
SMAD2	SMAD family member 2	4087	Known Cancer Gene
SMAD3	SMAD family member 3	4088	Known Cancer Gene
SMAD4	SMAD family member 4	4089	Known Cancer Gene
SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	6594	Exploratory Gene
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	6595	Exploratory Gene
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	6597	Known Cancer Gene
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	8467	Exploratory Gene
SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	56916	Exploratory Gene
SMARCAL1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	50485	Exploratory Gene
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	6598	Known Cancer Gene
SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	6599	Exploratory Gene
SMARCC2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	6601	Exploratory Gene
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	6602	Exploratory Gene
SMARCD2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	6603	Exploratory Gene
SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	6604	Exploratory Gene

SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	6605	Exploratory Gene
SMO	smoothened homolog (Drosophila)	6608	Known Cancer Gene
SNTG2	syntrophin, gamma 2	54221	Known Cancer Gene
SNX31	sorting nexin 31	169166	Known Cancer Gene
SOCS1	suppressor of cytokine signaling 1	8651	Known Cancer Gene
SOX17	SRY (sex determining region Y)-box 17	64321	Known Cancer Gene
SOX2	SRY (sex determining region Y)-box 2	6657	Copy Number Gene
SOX9	SRY-box 9	6662	Exploratory Gene
SPEN	spen homolog, transcriptional regulator (Drosophila)	23013	Exploratory Gene
SPOP	speckle-type POZ protein	8405	Known Cancer Gene
SS18	synovial sarcoma translocation, chromosome 18	6760	Exploratory Gene
STAG2	stromal antigen 2	10735	Exploratory Gene
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	6774	Known Cancer Gene
STK11	serine/threonine kinase 11	6794	Known Cancer Gene
STK19	serine/threonine kinase 19	8859	Exploratory Gene
STK3	serine/threonine kinase 3 (STE20 homolog, yeast)	6788	Exploratory Gene
STRADA	STE20-related kinase adaptor alpha	92335	Exploratory Gene
STXBP5L	syntaxin binding protein 5-like	9515	Known Cancer Gene
SUFU	suppressor of fused homolog (Drosophila)	51684	Known Cancer Gene
SUZ12	suppressor of zeste 12 homolog (Drosophila)	23512	Known Cancer Gene
TAB3	mitogen-activated protein kinase kinase kinase 7 interacting protein 3	257397	Copy Number Gene
TACC1	transforming, acidic coiled-coil containing protein 1	6867	Known Cancer Gene
TACC3	transforming, acidic coiled-coil containing protein 3	10460	Known Cancer Gene
TBC1D7	TBC1 domain family, member 7	51256	Copy Number Gene
TBX3	T-box 3	6926	Known Cancer Gene
TCF7	transcription factor 7 (T-cell specific, HMG-box)	6932	Known Cancer Gene
TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)	83439	Known Cancer Gene
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	6934	Known Cancer Gene
TEAD4	TEA domain transcription factor 4	7004	Exploratory Gene
TERT	telomerase reverse transcriptase	7015	Copy Number Gene
TET2	tet oncogene family member 2	54790	Known Cancer Gene

TEX15	testis expressed 15	56154	Known Cancer Gene
TFE3	transcription factor binding to IGHM enhancer 3	7030	Known Cancer Gene
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	7048	Known Cancer Gene
TLR3	toll-like receptor 3	7098	Copy Number Gene
TMEM173	transmembrane protein 173	340061	Exploratory Gene
TMPRSS2	transmembrane protease, serine 2	7113	Known Cancer Gene
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	8743	Copy Number Gene
TNKS	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase	8658	Exploratory Gene
TNKS2	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2	80351	Exploratory Gene
TOP1	topoisomerase (DNA) I	7150	Copy Number Gene
TP53	tumor protein p53	7157	Known Cancer Gene
TP63	tumor protein p63	8626	Exploratory Gene
TP73	tumor protein p73	7161	Copy Number Gene
TPK1	thiamin pyrophosphokinase 1	27010	Copy Number Gene
TPR	translocated promoter region (to activated MET oncogene)	7175	Known Cancer Gene
TRAF2	TNF receptor-associated factor 2	7186	Exploratory Gene
TRAF3	TNF receptor-associated factor 3	7187	Exploratory Gene
TRAF7	TNF receptor-associated factor 7	84231	Exploratory Gene
TRRAP	transformation/transcription domain-associated protein	8295	Exploratory Gene
TSC1	tuberous sclerosis 1	7248	Exploratory Gene
TSC2	tuberous sclerosis 2	7249	Exploratory Gene
TSHR	thyroid stimulating hormone receptor	7253	Known Cancer Gene
TSTD1	thiosulfate sulfurtransferase KAT, putative	100131187	Known Cancer Gene
TTK	TTK protein kinase	7272	Copy Number Gene
TUBD1	tubulin, delta 1	51174	Known Cancer Gene
U2AF1	U2 small nuclear RNA auxiliary factor 1	7307	Known Cancer Gene
VEGFA	vascular endothelial growth factor A	7422	Copy Number Gene
VHL	von Hippel-Lindau tumor suppressor	7428	Known Cancer Gene
VTI1A	vesicle transport through interaction with t-SNAREs homolog 1A (yeast)	143187	Known Cancer Gene
WDFY4	WDFY family member 4	57705	Known Cancer Gene
WDR5	WD repeat domain 5	11091	Exploratory Gene
WHSC1	Wolf-Hirschhorn syndrome candidate 1	7468	Exploratory Gene
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	54904	Exploratory Gene
WRN	Werner syndrome RecQ like helicase	7486	Exploratory Gene
WSB1	WD repeat and SOCS box-containing 1	26118	Copy Number Gene
WT1	Wilms tumor 1	7490	Known Cancer Gene

WWOX	WW domain containing oxidoreductase	51741	Copy Number Gene
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1	7515	Known Cancer Gene
YAF2	YY1 associated factor 2	10138	Copy Number Gene
YAP1	Yes-associated protein 1, 65kDa	10413	Exploratory Gene
ZC3H13	zinc finger CCCH-type containing 13	23091	Known Cancer Gene
ZEB2	zinc finger E-box binding homeobox 2	9839	Known Cancer Gene
ZNF132	zinc finger protein 132	7691	Known Cancer Gene
ZNF217	zinc finger protein 217	7764	Copy Number Gene
ZNF324	zinc finger protein 324	25799	Copy Number Gene
ZNF639	zinc finger protein 639	51193	Known Cancer Gene
ZNF703	zinc finger protein 703	80139	Exploratory Gene
ZNF704	zinc finger protein 704	619279	Copy Number Gene

Supplementary Table S5.2: Pathway names and member genes; pathway level alterations in cohort; number of genetic alterations in each pathway for individual samples.

Supplementary Table S5.3: List of SNVs, indels, gains and losses.

Chapter 6
Conclusion and Future Directions

6.1 Summary of Findings

As mentioned in the Introduction, this collection of studies with the theme "Neurofibromatosis Type 1 (NF1) and Breast Cancer (BC)" was initiated after seeing a series of women with NF1 and breast cancer at National Cancer Centre Singapore (NCCS) from 2006 to 2009. These patients under my care had aggressive HER2-positive disease that did not seem to respond to standard systemic therapies as well as in individuals without NF1 syndrome. This clinical observation triggered a literature search, which revealed limited data at the time of commencement of my candidature in 2011. The studies included in this thesis were conceived to address clinically relevant questions around the topic of neurofibromatosis type 1, the NF1 gene and breast cancer.

Since 2007, the higher risk of breast cancer in women with NF1 has been increasingly recognized, especially for the younger women under 50 years [1,2]. Data on the characteristics of BC in NF1 patients is still relatively sparse. Our group was the first to discover the higher frequency of HER2-positive, hormone receptor negative and grade 3 breast cancers in women with NF1, and also the first to genomically profile these NF1-associated breast cancers.

Over the course of my candidature, data from large-scale exome or genome sequencing studies led by TCGA, ICGC and METABRIC, have unravelled the heterogeneity and complexity of cancer genomes. Somatic *NF1* aberrations were detected in different sporadic tumours from individuals without NF1 syndrome (Source: The cBio Cancer genomics Portal; http://www.cbioportal.org). In my projects, we also aimed to explore the potential role of *NF1* and neurofibromin in sporadic breast cancers from patients without NF1. This included immunohistochemical staining of tissue micrroarrays, and targeted gene sequencing (with *NF1* in the gene panel) of poor prognosis breast cancers in premenopausal women. This study group was selected as there is a higher proportion of premenopausal breast cancers in Asia

compared to the West populations. It was proposed that *NF1* mutations may contribute to themore aggressive disease and poor prognosis of this younger aged group.

In this thesis, we started with a systematic overview in Chapter 1**, "**The NF1 gene revisited – from bench to bedside". This comprehensive review of the literature focused on the oncological aspects of the germline NF1 disorder, the biology of the NF1 gene and neurofibromin, tumours associated with NF1 as well as sporadic tumours harbouring somatic *NF1* aberrations in individuals without NF1 disorder. *NF1*, a tumour suppressor gene, plays a critical role in carcinogenesis. The gene product neurofibromin is a negative regulator of the Ras cellular proliferation pathway [3,4], and also exerts tumour suppression via other mechanisms[5]. During the course of the candidature, with the data that emerged from the large scale tumour sequencing projects, somatic *NF1* aberrations have been increasingly reported in various sporadic tumours, including brain, lung, breast, ovarian tumours as well melanomas and leukemias. *NF1* alterations appear to be associated with resistance to therapy and adverse outcomes in several tumour types [5]. Identification of a patient's germline or somatic *NF1* aberrations can be challenging, as *NF1* is one of the largest genes in the human genome (60 exons spanning over 350kb of genomic DNA), and in the absence of any mutation hotspots, has a myriad of possible mutations. Epigenetic factors may also contribute to inadequate levels of neurofibromin in cancer cells. To our knowledge, the efficacy of *NF1*-based therapeutic approaches has not been established. Clinical trials using various agents, including mTOR inhibitors, for mainly the plexiform neurofibromas in individuals with NF1, have to date not yielded any impressive results [5]. It is hoped that the emerging recognition of the role of *NF1* in sporadic cancers will lead to additional clinical trials exploring *NF1*-based treatments, such as MEK inhibitors, for various tumour types. Improved understanding of the implications of *NF1* aberrations is critical for the development of novel therapeutic strategies.

In Chapter 2, titled "Whole exome sequencing of multiple tumours from an NF1 patient"**,** apart from the germline *NF1* mutation (L847P), we demonstrated independent somatic *NF1* mutations in the 3 tumors from this patient (frameshift insertion in breast cancer (p.A985fs), missense mutation in malignant peripheral

nerve sheath tumour (p.G23R**)**, and inframe deletion in dermal neurofibroma (p.L1876del-Inf)), indicating that a second hit in *NF1* gene resulting in its loss of function is likely to be critical for tumor formation [6]. This is the classic scenario of a tumour suppressor as first demonstrated in the retinoblastoma gene by Knudson. Each of the three tumours had a distinct genomic profile with mutually exclusive mutations in different genes. Copy number analysis revealed multiple copy number alterations in the breast cancer and the MPNST, but not the benign neurofibroma. Germline loss of chromosome 6q22.33, which harbors 2 potential tumor suppressor genes, *PTPRK* and *LAMA2*, was also identified; this may increase tumour predisposition further. In the background of NF1 syndrome, although second-hit *NF1* mutation is critical in tumorigenesis, different additional mutations are required to further drive the formation of different tumors.

The aim of Chapter 3, "Comprehensive case series of BCs in women with NF1 with molecular insights into its aggressive phenotype" was to improve the understanding of NF1-associated breast cancer, given the increased risk of breast cancer in this tumour predisposition syndrome and the limited available data. We identified 18 women with NF1 and breast cancer at our institution, and performed next generation sequencing on DNA from the available blood and breast cancer specimens. Expression of neurofibromin in the NF1-associated breast cancers was evaluated using immunohistochemistry. Compared with 7132 breast cancers in patients without NF1 from our institutional database, there was a higher frequency of grade 3 (83.3% vs 45.4%, p=0.005), oestrogen receptor (ER) negative (66.7% vs 26.3%, p<0.001) and human epidermal growth factor receptor 2 (HER2) positive (66.7% vs 23.4%, p<0.001) tumours among NF1 patients. Overall survival was inferior in NF1 patients compared with the non-NF1 breast cancers in multivariable analysis (HR 2.25, 95% CI, 1.11 to 4.60; p = 0.025). Apart from germline *NF1* mutations (11/16 detected; 69%), somatic mutations in *TP53* (8/10; 80%), second-hit *NF1* (2/10; 20%), somatic mutations in *KMT2C* (4/10; 40%), *KMT2D* (2/10; 20%), and *PIK3CA* (2/10; 20%) were observed. Immunohistochemical expression of neurofibromin was seen in the nuclei and/or cytoplasm of all specimens, but without any consistent pattern in its intensity or extent. This comprehensive series of NF1-associated breast cancers suggests that their aggressive features are related to germline *NF1* mutations in cooperation

with somatic mutations in *TP53, KMT2C* and other genes. The high frequency of TP53 mutations in BC of NF1 syndrome patients was particularly striking.

Chapter 4, “Immunohistochemical expression of neurofibromin in sporadic breast cancers” explores the potential role of *NF1* and neurofibromin in sporadic breast cancers from patients without NF1. There is limited data on expression of neurofibromin in breast cancer, and the clinical relevance of neurofibromin deficiency in sporadic breast cancer is unclear. We hypothesised that loss of expression of neurofibromin, a tumour suppressor, will be associated with overexpression of pAkt and pMAPK, both downstream in the PI3K-MAPK pathway, and this contributes tp the resulting worse outcomes. The expression of neurofibromin was initially evaluated through immunohistochemistry on microarrayed cores obtained from 314 stage 1-3 breast cancer specimens diagnosed between 2000 and 2002. “Positive” expression of neurofibromin was defined as nuclear and cytoplasmic staining in 10% or more of tumour cells. Positive expression of neurofibromin, as defined above, was seen in 44.6% (140/314) of tumours. “Negative” expression of neurofibromin was associated with high tumour grade ($p<0.001$), hormone receptor negativity ($p<0.001$), lymph node positivity ($p=0.041$) and larger tumour size ($p=0.031$). On multivariate analysis, lack of neurofibromin as defined above, was an independent predictor of relapse and death for triple negative cancers (hazard ratios 3.33, $p=0.011$ and 2.94, $p=0.026$ respectively), but not in the luminal and HER2 positive immunohistochemical subtypes. To validate this finding, the expression of neurofibromin was subsequently evaluated in tissue microarrays with a total of 594 triple negative breast cancers diagnosed from 1993 to 2011. No association between neurofibromin expression survival outcomes was found in the validation set. Immunohistochemistry with currently available antibodies may be suboptimal for assessing deficiency or dysfunction of neurofibromin. The epitope for these antibodies is restricted to a limited region of the large neurofibromin protein, and cannot detect abnormal neurofibromin from mutations outside their specific epitopes. Novel methods such as mass-spectrometry-based proteomic analyses may potentially be superior for this purpose.

In Chapter 5, “Elucidating therapeutic molecular targets in premenopausal Asian women with recurrent breast cancers”, targeted sequencing was performed using

FFPE specimens of the breast primary from a separate cohort of premenopausal patients who subsequently relapsed after initial diagnosis of non-metastatic disease. The most prevalent alterations included *TP53* (65%). *PIK3CA* (32%), *GATA3* (29%), *ERBB2* (27%), *MYC* (25%) and *KMT2C* (21%). The frequency of *NF1* mutation was 2%, somewhat similar to the large scale breast cancer sequencing studies. Detecting changes in copy number of the *NF1* gene in formalin-fixed paraffin-embedded specimens was not feasible.

6.2 Suggestions for Future Work

The important role of *NF1* in various tumours is increasingly recognized, but challenges remain in the detection of *NF1* aberrations as well as deficiency and dysfunction of neurofibromin [5]. A multi-omics approach incorporating the DNA, RNA and proteins from each tumour can help to provide better insights, as epigenetic factors can influence the expression of key molecules in the complex cancer pathways. Analysis of copy number changes and more complex rearrangements in addition to mutations alone will also provide more comprehensive information on the tumour profile. Novel methods such as mass spectrometry-based proteomic analyses may potentially be promising, given the limitations of immunohistochemistry from our experience.

Future studies should ideally aim to capture serial specimens using a longitudinal study, since the tumour genome is constantly evolving. While the frequency of *NF1* mutations in primary breast cancers is generally less than 5%, recent data suggest they are enriched in the metastatic specimens, highlighting the role of *NF1* in the metastatic process [7]. Consideration should also be given to the epigenetic and tumour microenvironment (including the immune ecosystem and the microbiome) in future studies, in addition to functional work to investigate the biological significance of alterations in *NF1*.

Last but not least, we hope that these new findings can be translated to better treatment outcomes for both patients with germline NF1 syndrome, as well as patients with sporadic tumours harbouring *NF1* alterations. Although targeting the

Ras pathway with a number of trials testing MEK inhibitors is the current focus, inhibiting a single checkpoint may lead to activation of compensatory negative feedback pathways. Blockade of multiple targets may not be feasible in clinical practice due to the additive toxicities. The role of immunotherapy in these tumours may be worth exploring, given the impressive breakthroughs with immune checkpoint blockade in several tumour types, including the recent report of impressive response rates in desmoplastic melanomas with *NF1* mutations and high mutation load [8].

6.3 Concluding Remarks

In conclusion, *NF1*, a tumour suppressor gene, plays a key role in various sporadic tumours including breast cancers, beyond the germline NF1 tumour predisposition disorder. *NF1* is one of the significantly mutated genes in various sporadic tumours from patients without NF1. In spite of recent progress with next generation sequencing technology, challenges remain with the detection of *NF1* alterations and the interpretation of the functional significance. Improved understanding of the biology will hopefully lead to better treatments and outcomes not only for patients with NF1 syndrome, but also patients with tumours harbouring *NF1* somatic aberrations.

6.4 References

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