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REVIEW

BRAF gene: From human cancers to developmental syndromes

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KEYWORDS

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Abstract The *BRAF* gene encodes for a serine/threonine protein kinase that participates in the MAPK/ERK signalling pathway and plays a vital role in cancers and developmental syndromes (RASopathies). The current review discusses the clinical significance of the *BRAF* gene and other members of RAS/RAF cascade in human cancers and RAS/MAPK syndromes, and focuses the molecular basis and clinical genetics of *BRAF* to better understand its parallel involvement in both tumourigenesis and RAS/MAPK syndromes—Noonan syndrome, cardio-facio-cutaneous syndrome and LEOPARD syndrome.

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Contents

1. Introduction

The *BRAF* gene on chromosome 7 (7q34) encodes the *BRAF* protein, which participates in the MAP kinase/ERK signalling pathway. This pathway regulates important cell functions including cellular growth, differentiation, proliferation, senescence and apoptosis (Peyssonnaux and Eychene, 2001). Different variant products of the *BRAF* gene have been reported as both activating and silencing the RAS/MAPK pathway (Rajagopalan et al., 2002; Davies et al., 2002; Makita et al., 2007; Cho et al., 2006; Aoki and Matsubara, 2013). Additionally, an increase in protein expression or activity can disturb the Ras–MAPK signalling pathway, which in turn can result in different developmental disorders such as Noonan syndrome (NS), cardio-facio-cutaneous (CFC) syndrome, Costello syndrome, and different types of human cancers. Similar to *BRAF*, germline mutations in the *PTPN11*, *SOS1*, *RAF*, and *KRAS* genes also influence the RAS/MAPK signalling pathway and contribute to the development of NS in approximately 70% of patients (Rodriguez-Viciana et al., 2006; Tartaglia et al., 2001; Romano et al., 2010).

In addition to germline mutations, *BRAF* somatic mutations have been reported in Langerhans cell histiocytosis (Badalian-Very et al., 2010; Satoh et al., 2012), Erdheim–Chester disease (Haroche et al., 2012), and lung, colon, thyroid, and melanoma cancers, as well as in non-Hodgkin lymphoma (Davies et al., 2002; Makita et al., 2007; El-Osta et al., 2011; Brose et al., 2002; Ohtake et al., 2011; Niihori et al., 2006). More than 80% mutations in the *BRAF* gene have been reported with varied frequencies. For instance, ~67% of melanomas exhibit *BRAF* mutations, whereas 30% of other tumours, such as thyroid cancer (15%), lung cancer (3%) and colorectal cancer (12%), present with *BRAF* mutations (Davies et al., 2002; Clancy et al., 2013; Puxeddu and Filetti, 2013). Among all cancers, V600E, has been examined in 66% of malignant melanomas (Rajagopalan et al., 2002; Ibrahim and Haluska, 2009), and specifically localised to the serine/threonine kinase domain. Additionally, somatic mutations resulting in *BRAF* heterozygous variants in kinase domain, such as G1382T (R461I), T1385G (I462S), G1388A (G463E), and A1798G (K600E), have been identified in colorectal cancer patients (Wan et al., 2004). According to some recent reports of the past 2–3 years, somatically acquired T1799A (V600E) mutation has been characterised in hairy cell leukaemia and related lymphoproliferative disorders (Xi et al., 2012; Blombery et al., 2012). However, the somatic mutations with G465V (exon 11) and L596R (exon 15) amino acid substitutions have been characterised in human lung adenocarcinomas (Naoki et al., 2002). An in-frame fusion of the *AKAP9* gene (exons 1–8) to the *BRAF* gene (exons 9–18), which occurs through a paracentric inversion of chromosome 7, has been preferentially recognised in radiation-induced papillary carcinomas, compared to *BRAF* point mutations (Ciampi et al., 2005). Moreover, two novel *BRAF* gene fusions—*KIAA1549-BRAF* and *FAM131B-BRAF*—have been identified in pilocytic astrocytomas (Cin et al., 2011; Sievert et al., 2009), and one (*PAPSS1-BRAF*) in melanoma (Hutchinson et al., 2013).

At the level of the BRAF protein, the amino acid variations observed as a result of *BRAF* mutations—R461I, I462S, G463E, G463V, G465A, G465E, G465V, G468A, G468E, N580S, E585K, D593V, F594L, G595R, L596V, T598I, V599D, V599E (V600E), V599K, V599R, V600K, and A727V—are mostly clustered in two regions: the glycine rich P loop region and the activation domain—protein kinase domain (Fig. 1) (Wan et al., 2004). These mutations change the activation peptide from an active state to an inactive state. For instance, as has been published previously, the charge density of the phenyl ring of Phe467 in the P loop interacts with the aliphatic side chain of vicinal Val599 (Val600). However, in the case of substitution of the medium-sized hydrophobic Val with a bulky charged moiety (Glu, Asp, Lys, or Arg), as is often found in human cancers, the interactions that preserve the DFG motif in an inactive conformation are destabilised, hence, the activation segment flips stereochemically into an inactive position (Wan et al., 2004; Bollag et al., 2012). Depending on the nature of the mutation, the activity of the BRAF protein towards MEK/ERK may also vary, thereby resulting in diversity of the underlying cellular pathways.

The present review summarises the role of the *BRAF* gene in different cancers and developmental disorders (NS, CFC

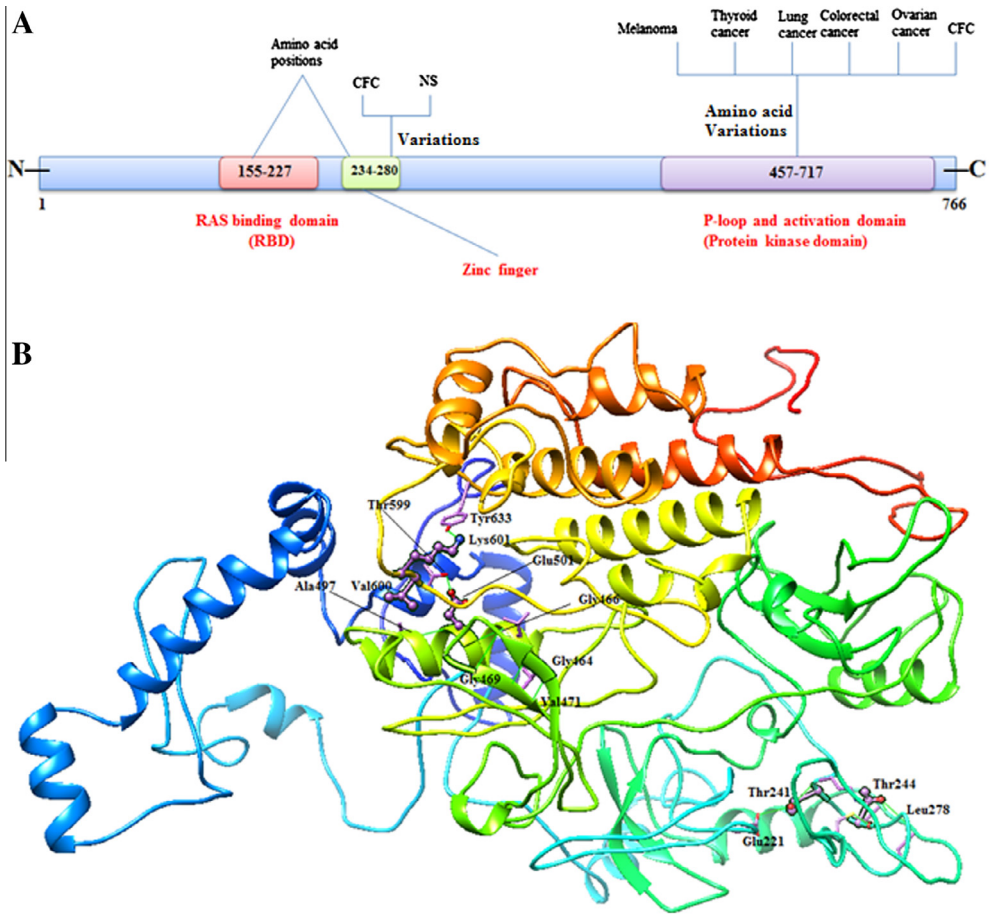

Figure 1 Protein domains connecting the cancers and RASopathies, and 3D structure of the BRAF protein. (A) BRAF mutations common to kinase domain (457–717 amino acids) are characterised mostly in melanoma, colorectal cancer, lung cancer, thyroid cancer and ovarian cancer, and CFC. (B) 3D structure of the BRAF protein with highlighted residues at the 241, 257, 469, 499 and 600 positions—the most common sites for amino acid substitutions, screened in NS, CFC and cancer diseases.

and LEOPARD syndromes). For this purpose, different electronic databases like PubMed, OMIM and UniProt were searched with the keywords "BRAF mutation", "BRAF V600E", "cancer BRAF", "BRAF MEK", "KRAS BRAF", "BRAF NRAS", "BRAF CFC", and "BRAF RASopathies" for extensive literature review.

2. *BRAF* mutations in various types of cancers

Cancer results from mutations in oncogenes (Hussain et al., 2014; Gao et al., 2014), including *BRAF* (Fig. 2), that provide alternative pathways for cellular processes, such as cell proliferation, differentiation and death (Peyssonnaux and Eychene, 2001). Dysregulated binding of RAS to BRAF and MEK proteins in the Ras/Raf/MEK/ERK cascade (Fig. 3A)—as a result of *BRAF* mutations—causes spontaneous signalling to downstream kinases, resulting in the overactivation of downstream signalling by MEK and ERK (Peyssonnaux and Eychene, 2001; Namba et al., 2003). In more than 90% of the cases of *BRAF* mutations, the thymine residue at the nucleotide position 1799 is substituted with an adenine residue (Tan et al., 2008), and extensively reported in papillary thyroid tumours (40%) Chappell et al., 2011; Pritchard et al., 2007, melanoma (< 50%) Chappell et al., 2011; Tan et al., 2008; Ascierto et al., 2012, colorectal tumours (10%) Cho et al., 2006, prostate tumours (10%) Cho et al., 2006 and non-small-cell lung cancers (Li et al., 2006; Benlloch et al., 2006; Deng et al., 2004; Gear et al., 2004; Maldonado et al., 2003; Puxeddu et al., 2004; Elisei et al., 2008). In addition to this, Gayane et al. confirmed the occurrence of the *BRAF* V600E mutation in Langerhans cell histiocytosis patients (~57%) Badalian-Very et al., 2012. Enrico et al. used parallel sequencing to determine that the V600E mutation is the most probable cause of hairy cell leukaemia (Tiacci et al., 2011).

Various *BRAF* mutations have been observed in 66% of malignant melanomas, and all of these mutations occurred in the serine/threonine kinase domain, resulting in the single variant V600E (Davies et al., 2002). Such mutations increase the kinase activity and transform the mutated protein in NIH3T3 cells (Davies et al., 2002). A few experimental studies have documented that these mutations are not observed in cancer cell lines derived from neuroblastomas, leukaemias, and lymphomas, or pancreatic, prostate, bladder, testicular, renal and cervical carcinomas (Davies et al., 2002; Vredeveld et al., 2012).

Because the over-activation of the RAS–RAF signalling by oncogenic *BRAF* has been associated with many different types of malignancies (Brose et al., 2002; Nikiforova et al.,

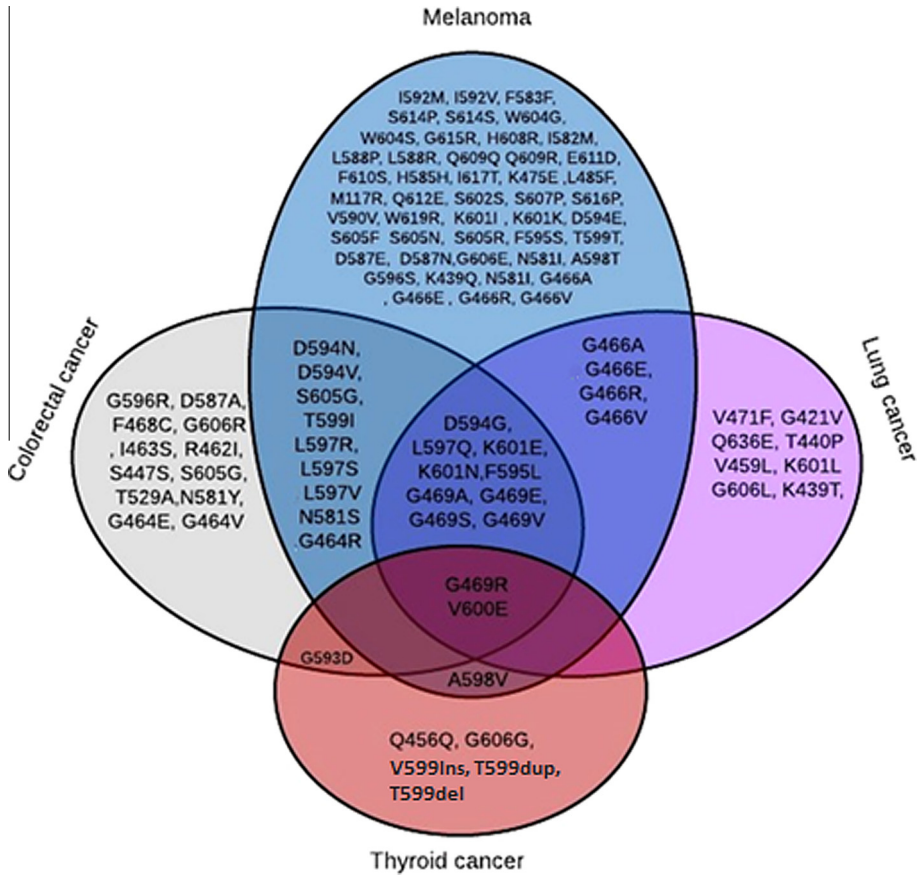

Figure 2 Venn diagram to show *BRAF* mutations (amino acid variations), characterised in different cancer types. The highest number of mutations is seen in melanomas, indicated in blue colour circle. The smaller number of mutations is observed in thyroid cancer, shown in red colour circle. However, mutations observed in more than one type of cancer are shown in the overlapping regions. For example, the G469R and V600E mutations are characterised in all 4 types of cancers, shown by the overlapping region.

2003; Wang et al., 2003; Park et al., 2013), it has been suggested that a decrease in the activity of oncogenic *BRAF* would be beneficial, thereby making this gene a potential therapeutic target in oncology (Davies et al., 2002; Nikiforova et al., 2003; Bhatia et al., 2009).

3. Papillary thyroid cancer (PTC)

BRAF, an isoform of the RAF kinase in the MAPK pathway, mutations have been reported in a variety of human cancers (Fig. 2) Brose et al., 2002; Hussain et al., 2012; Benlloch et al., 2006; Bosmuller et al., 2013; Ul-Haq et al., 2012 including papillary thyroid cancer (PTC), which accounts for 80% of thyroid malignancies (Xing et al., 2005). Papillary thyroid carcinomas harbour point mutations in the *BRAF* and *RAS* genes that underlie the MAPK pathway (Fig. 3A) Nikiforov, 2011. The estimated occurrence of *BRAF* mutations in PTC patients ranges from 29% to 83%. Many clinical studies have been conducted to determine the behaviour of such mutations, particularly the most common V600E mutant, in the majority of sporadic PTC cases (Jo et al., 2006). Additionally, the association of the *BRAF* V600E mutation with the transcription co-activator Yes-associated protein 1 (YAP1) has recently been found to be responsible for tumour progression in PTC and anaplastic thyroid cancer (Lee et al., 2013). Another study in the same context supports the possible association between the expression patterns of the *PKM2* gene and *BRAF* mutations that mediate PTC (Feng et al., 2013). According to recent findings, the intriguing occurrence of the K601E and V600E mutations in the *BRAF* gene has been found in multifocal papillary thyroid carcinoma (mPTC) and conventional PTC, respectively (Kim et al., 2013).

Another recent study concerning the conversion of metastatic papillary thyroid carcinoma (PTC) into primary squamous cell carcinoma (SCC) has suggested that PTC can reoccur in the cervical lymph nodes, as reported in an 86-year-old woman 10 years after total thyroidectomy (Lee et al., 2013). After thyroidectomy, the failure of radioiodine treatment may cause a recurrence of PTC and increased morbidity and mortality. Hence, the more aggressive behaviour of the *BRAF* V600E mutation results in the need for a stronger treatment for PTC patients to overcome the recurrence of tumours (Xing et al., 2005). The independent role of the *BRAF* V600E mutation as a potential marker in the risk stratification of papillary thyroid cancer (PTC) patients can help to efficiently treat patients and prevent recurrent disease (Xing, 2007). Similar findings have also indicated a close relationship between PTCs carrying the V600E mutation and high expression levels of VEGF (vascular endothelial growth factor), which in turn is associated with aggressive tumour

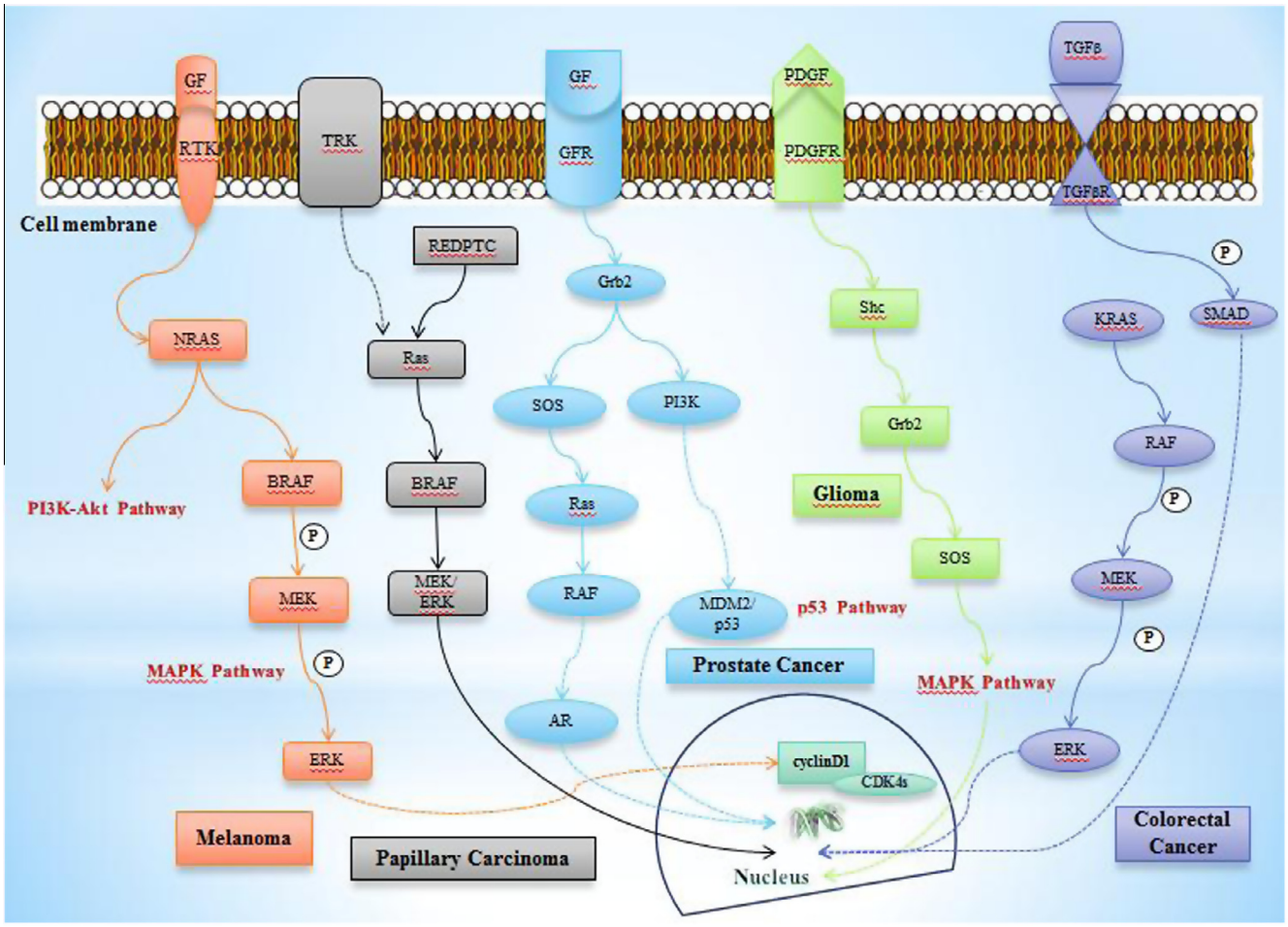

Figure 3 Schematic diagram showing the RAS–RAF signalling pathway with different cancers (A) and RASopathies (B). In melanoma, in red colour, *RAS* and *BRAF* mutations activate both effector pathways: Raf–MEK–ERK (*BRAF* gene) and PI3K–Akt signalling. In thyroid cancer (papillary carcinoma), in grey colour, both *RAS* and *RAF* (*BRAF*) play a combined critical role in proliferation. Molecular cascade underlying prostate cancer (turquoise) underlies both MAPK and p53 pathways. The glioma involves PDGF, PDGFR, Sch, Grb2, SOS, Ras, Raf and MAPK. In colorectal cancer, indicated in blue colour, both TGF-β and MAPK signalling pathways play a critical role. RAS/RAF signalling transduction for developmental syndromes (NS, CFC and LEOPARD) is indicated in B.

characteristics such as tumour size, extrathyroidal extension and tumour stage (Nikiforov, 2011). At the protein level, V600 in the glycine-rich loop of BRAF forms a hydrophobic interaction with P468, which stabilises the DFG motif by preventing the phosphoryl transfer to the DFG triad, finally locking it in an inactive conformation. As a result of the V600E variation, the substitution of valine with glutamic acid disrupts that hydrophobic interaction and induces BRAF phosphorylation, which is associated with an up to 700-fold increase in the MEK–ERK signalling (Ziai and Hui, 2012).

Fine-needle aspiration (FNA) of biopsy material (thyroid nodules) is the most reliable way to preoperatively diagnose thyroid carcinomas carrying the *BRAF* V600E mutation. In the United States, approximately 60–70% of the biopsied thyroid nodules are characterised as benign, while 4–10% are categorised cytologically malignant (Adeniran et al., 2011). The nature of the remaining 20–30% of the nodules cannot be diagnosed due to cytological uncertainty. In such equivocal cytological contexts, testing FNA specimens for the *BRAF* V600E mutation has facilitated the definitive diagnosis of PTC (Adeniran et al., 2011; Rowe et al., 2006).

In paediatric PTC, appropriate surgery with adjuvant radioiodine ablation is often enough to cure the disease, even if the disease is quite extensive. This is because *BRAF* mutations are rarely found in paediatric PTC, or these mutations appear to cause the poor prognosis of PTC. Moreover, a high occurrence of the T1799A nucleotide mutation, which results in the V600E mutation (previously known as V599E), in the lymph node-metastasised PTC tumours, it may reflect the *de novo* development of thyroid cancer cells of increased malignancy. In another report concerning the lymph node metastasis of PTC, a novel mutation—the deletion of codon 601—appeared to be the cause of the genetic alteration underlying the progression of PTC cells in the lymph nodes (Vasko et al., 2005).

4. *BRAF* gene and malignant melanoma

According to recent statistics released by the National Cancer Institute of the NIH (http://www.cancer.gov/cancertopics/types/melanoma), 76,100 new cases of melanoma have been reported in 2014. Massachusetts General Hospital documented

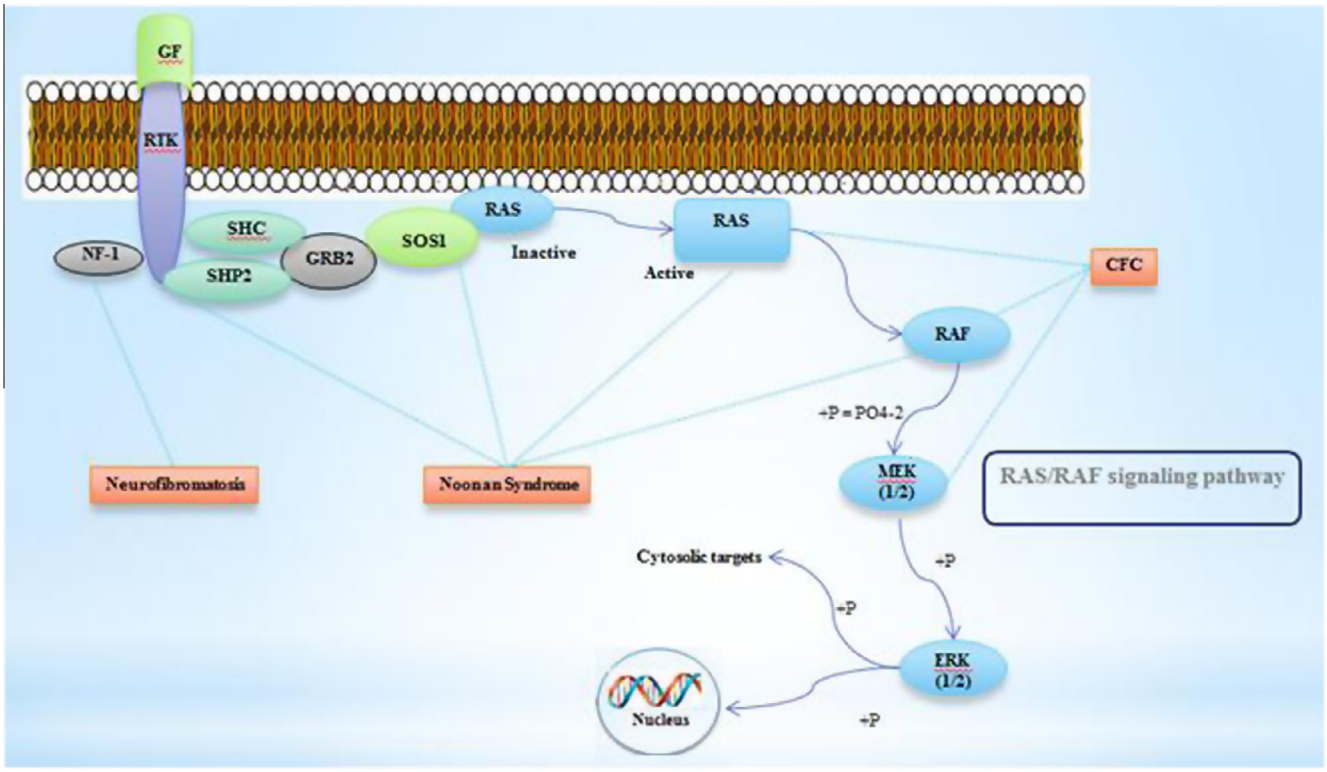

Figure 3 (*continued*)

a male approximately 68 years old who developed metastatic melanoma to the groin and pelvis 11 years after a melanoma on his right leg was excised (Sullivan et al., 2013). Additionally, in 2008, the highest (8420) estimated deaths in the United States were due to metastatic melanoma (Jemal et al., 2008). In the same year, Wajapeyee et al. (2008) used genomewide RNA interference screening to identify 17 factors—F-box protein (FBXO31)—necessary for oncogenic BRAF to regulate senescence in primary fibroblasts and melanocytes.

Dermatologists are typically on the forefront to identify patients with increased risk of melanoma due to genetic predisposition; however, most melanoma cases are sporadic and result from moderate genetic risk factors (Udayakumar et al., 2010). The strongest genetic risks for melanoma development have been observed as a result of genetic variations in the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene located on chromosome 9p21 (Nelson and Tsao, 2009). Studies have also identified a high frequency of *BRAF* somatic missense mutations in malignant melanoma. In addition, *MC1R* variants are strongly associated with *BRAF* mutations in non-CSD (chronic solar-damaged) melanoma (Fargnoli et al., 2008). Thus, high risk for melanoma is associated with the both the *MC1R* and *BRAF* genes. However, *BRAF* mutations have also been rarely detected in CSD melanomas (18.2%), as well as in acral (15.5%) and mucosal (12.5%) melanomas (Si et al., 1990).

More than 90% of the *BRAF* mutations in human malignant melanomas involve codon 600, and 80% of these mutations are comprised of the V600E mutation. Nevertheless, V600K is the second most common mutation, constituting 14–28% of the *BRAF* mutations that underlie melanoma (Forschner et al., 2013). From one recent study, three novel mutations in exon 11 of *BRAF*—G442S, W450Stop and I457T—have been identified in melanoma (acral and mucosal) in addition to the eight previously reported mutations in exon 11 and 15: G466R, D594E, D594G, V600E, V600K, K601E, W604Stop and S616F (Si et al., 1990).

From the literature, the BRAF protein has three AKT phosphorylation sites: Thr439, Ser428 and Ser364. *In vitro* substitution of the Thr439 residue with an Ala residue leads to BRAF activation through the loss of AKT-induced inhibition of RAF1 phosphorylation. Hence, the detection of K438T and K438Q in melanoma, similar to Thr439, is likely to inhibit AKT-dependent phosphorylation (Brose et al., 2002). Similarly, the presence of Ser428, Ser364 and the aforementioned novel mutations (G442S, W450Stop and I457T) (Si et al., 1990) in the vicinity of Thr439 are suggested to play a role in tumourigenesis (Brose et al., 2002).

5. Involvement of the *BRAF* and *KRAS* genes in ovarian cancer

During the past few years, patients with epithelial ovarian cancer have been treated identically in clinical trials and in pathological practise (Gershenson, 2013). The development of a 2-tier system for serous carcinomas, with low and high grades, is now generally accepted (Kohn and Hurteau, 2013). This simple approach, which is based on biological evidence, proposes that both tumours develop through different pathways. With infrequent mitotic figures, low-grade serous carcinomas, such as invasive micropapillary serous carcinoma (MPSC), show low-grade nuclei and are believed to evolve from adenofibromas or cystadenomas, which are borderline in the tumour-serous carcinoma sequence, via mutation in

the *KRAS*, *BRAF*, or *ERBB2* genes (Vang et al., 2009). Mutations in the *KRAS*, *BRAF*, or *ERBB2* genes result in the upregulation of MAPK, which in turn regulates uncontrolled proliferation in atypical proliferative serous tumours (APST). Serous cystadenomas found adjacent to APSTs have been found to have identical *KRAS* or *BRAF* mutations, suggesting that the presence of APSTs near the cystadenomas may support the regulation of serous carcinomas (Vang et al., 2009).

While *BRAF* V600E has been reported to be prevalent in 33% of tumours in the past, recent findings exhibited only 2% low-grade serous mutations (Ziai and Hui, 2012). Conversely, in low-grade ovarian serous carcinoma patients, the correlation between *BRAF* and *KRAS* has not yet been explored. Additionally, unlike previous findings, one report discussed the low frequency characterisation of *KRAS* and *BRAF* mutations in advanced stage low-grade serous carcinomas (Wong et al., 2010). Recently, 20–40% of low-grade serous carcinomas have been characterised with *KRAS* mutations, while *BRAF* mutations are found in approximately 5% of this subtype (Gershenson, 2013). In the literature, most of the *BRAF* mutations have been identified using DNA-based techniques; however, Bösmüller et al. differentiated the *BRAF* V600E mutation using mutation-specific monoclonal antibody (VE1) specific for the *BRAF* V600E. This ability to immunostain the *BRAF* V600E mutant protein in serous ovarian tumour samples with low epithelial content demonstrates the practical usefulness of mutation-specific recognition by an antibody (VE1) (Bosmuller et al., 2013).

6. Population based specificity of *BRAF* mutations in prostate cancer

Prostate cancer, the most common cancer type in Western Europe and USA, has variable incidence of *BRAF* and/or *KRAS* mutations among different races (Ren et al., 2012; Shen et al., 2010). Three reports have recorded *BRAF* mutations in prostate carcinomas, and one of these showed no *BRAF* mutations in the American and German populations. However, 10.2% of prostate carcinomas have been identified as having *BRAF* mutations in the Korean population (Cho et al., 2006). In addition, 10% of prostate cancer cases have been characterised as having an activating *BRAF* mutation in the Asian population (Kollermann et al., 2010). This difference in *BRAF* mutation frequency has been characterised in many different ethnic backgrounds. According to recent evidence, a gain in the *RAF* gene copy number resulted in the activation of the RAS/RAF/MEK/ERK pathway was found to be the main contributing element in the regulation of prostate cancer in a Chinese population (Ren et al., 2012). However, in another report of Chinese patients, *KRAS* mutations at codons 12 and 13 were found in prostate cancer patients while no *BRAF* mutation was observed at codon 600 (Shen et al., 2010). Caucasian patients had results similar to the Chinese patients, suggesting that *BRAF* mutations are rarely found in prostate cancer and are not connected with tumour progression. Nevertheless, recurrent gene fusions have shown that the *SLC45A3-BRAF* and *ESRP1-RAF1* fusions drive the molecular events occurring in advanced prostate and gastric cancers, as well as melanoma (Palanisamy et al., 2010). Subsequently, Kollermann et al. have argued that the emergence and the increased usage of BRAF-targeted therapies may have limited efficacy in treating prostate cancer patients (Kollermann et al., 2010).

7. Lung cancer

In the United States, approximately 150,000 lives are lost to lung cancer each year. The therapies that are used to treat lung cancer only support the survival of 15% of patients for a period of 5 years (Naoki et al., 2002). According to the National Cancer Institute of the NIH (www.cancer.gov/cancertopics/types/lung), it is estimated that 159,260 deaths will occur from lung cancer (both non-small cell and small cell) in 2014. Although specific therapies have been developed to target the molecular alterations that lead to cancer, new therapeutic elements are still being developed based on genome-wide screening for genetic alterations to reduce the cancer death rate (Brose et al., 2002).

Molecular mechanisms underlying lung cancer involve the *KRAS*, *BRAF*, *c-erbB-2* and *EML4-ALK*, and inactivation of tumour suppressor genes, including *p16INK4a*, and *RASSF1*. Recent genomic studies have identified the genetic changes that lead to activating mutations in the *BRAF*, *HER2*, and *KRAS* genes in lung adenocarcinomas, and these mutations can be used as potential therapeutic targets in clinical decision-making (Cardarella and Johnson, 2013). *BRAF* mutations are found in 4% of non-small cell lung cancers (NSCLCs), and half of these mutations are non-V600E (Cardarella et al., 2013). Several other reports documented the identification of *BRAF* mutations in both small-cell and non-small-cell lung cancer pathogenesis. In one such report, two *BRAF* mutations, one in exon 11 (G465V) and one in exon 15 (L596R), have been observed in 127 primary lung adenocarcinoma specimens (Naoki et al., 2002). In another report, K438Q (exon 11), K438T (exon 11), and K600E (15 exon) mutations were found to be essential for AKT phosphorylation at Thr439 (Brose et al., 2002). However, in addition to the Thr439 residue, two additional sites of AKT phosphorylation, Ser364 and Ser428, were found to regulate this multistep mechanism. Hence, the target specific therapies for V600E are inadequate for immediate clinical use in lung cancer.

8. Colorectal cancer

Colorectal cancer (CRC) is one of the leading causes of cancer morbidity and mortality, and is the third most common malignant tumour in the western world (Yuan et al., 2013; Siegel et al., 2013). Genetic aberrations in the form of chromosomal abnormalities, genetic mutations, and epigenetic alterations regulate the development of colorectal cancer, which is a multistep phenomenon involving several genes (*KRAS*, *BRAF*, *MEK*, *ERK* and *c-Fos*) that mediate proliferation, differentiation, apoptosis, and angiogenesis.

Hypermethylation of the *MLH1* promoter region results in the loss of MLH1 protein expression and microsatellite instability (MSI) (Simpkins et al., 1999); this mutation leads to hereditary non-polyposis colorectal cancer (HNPCC), which represents 3–6% of the total burden of colorectal cancer (Bettstetter et al., 2007). The clinical associations of the combined MSI/BRAF subgroups in colorectal cancer were investigated as tumour molecular biomarkers for prognostic risk stratification (Lochhead et al., 2013). In addition, *KRAS* and

Table 1 BRAF variations characterised in NS, CFC and LEOPARD patients.

Amino acid variation	Syndrome	BRAF protein domain	References
T241M	NS	Zinc finger (Phorbol-ester/DAG-type)	Sarkozy et al. (2009)
T241R	NS	Zinc finger (Phorbol-ester/DAG-type)	Sarkozy et al. (2009)
W531C	NS	Protein kinase	Sarkozy et al. (2009)
L597V	NS	Protein kinase	Sarkozy et al. (2009)
T241P	CFC & LEOPARD	Zinc finger (Phorbol-ester/DAG-type)	Schulz et al. (2008) and Sarkozy et al. (2009)
T244P	CFC	Zinc finger (Phorbol-ester/DAG-type)	Schulz et al. (2008)
L245F	CFC	Zinc finger (Phorbol-ester/DAG-type)	Sarkozy et al. (2009)
A246P	CFC	Zinc finger (Phorbol-ester/DAG-type)	Niihori et al. (2006), Sarkozy et al. (2009) and Schulz et al. (2008)
Q257R	CFC	Zinc finger (Phorbol-ester/DAG-type)	Rodriguez-Viciana et al. (2006), Niihori et al. (2006), Sarkozy et al. (2009) and Schulz et al. (2008)
Q262K	CFC	Zinc finger (Phorbol-ester/DAG-type)	Schulz et al. (2008)
E275K	CFC	Zinc finger (Phorbol-ester/DAG-type)	Sarkozy et al. (2009)
S467A	CFC	Nucleotide binding (ATP)	Rodriguez-Viciana et al. (2006)
F468S	CFC	Nucleotide binding (ATP)	Rodriguez-Viciana et al. (2006) and Schulz et al. (2008)
G469E	CFC & colon cancer	Nucleotide binding (ATP)	Rodriguez-Viciana et al. (2006) and Schulz et al. (2008)
L485F	CFC	Protein kinase	Rodriguez-Viciana et al. (2006), Niihori et al. (2006) and Sarkozy et al. (2009)
K499E	CFC	Protein kinase	Rodriguez-Viciana et al. (2006), Niihori et al. (2006) and Schulz et al. (2008)
K499N	CFC	Protein kinase	Sarkozy et al. (2009) and Schulz et al. (2008)
E501G	CFC	Protein kinase	Rodriguez-Viciana et al. (2006) and Niihori et al. (2006)
E501K	CFC	Protein kinase	Rodriguez-Viciana et al. (2006), Niihori et al. (2006) and Sarkozy et al. (2009)
L525P	CFC	Protein kinase	Sarkozy et al. (2009)
W531C	NS	Protein kinase	Sarkozy et al. (2009)
N580D	CFC	Protein kinase	Schulz et al. (2008)
N581D	CFC	Protein kinase	Rodriguez-Viciana et al. (2006), Niihori et al. (2006) and Schulz et al. (2008)
F595L	CFC & colon cancer	Protein kinase	Davies et al. (2002), Rodriguez-Viciana et al. (2006), Sarkozy et al. (2009) and Schulz et al. (2008)
G596V	CFC	Protein kinase	Rodriguez-Viciana et al. (2006)
T599R	CFC	Protein kinase	Sarkozy et al. (2009)
K601Q	CFC	Protein kinase	Sarkozy et al. (2009)
D638E	CFC	Protein kinase	Sarkozy et al. (2009)
Q709R	CFC	Protein kinase	Sarkozy et al. (2009)

BRAF mutations are suggested to be poor prognostic factors in CRC patients with synchronous liver metastasis (Huang et al., 2013; Umeda et al., 2013). Screening for both *BRAF* mutations in codon 600 and *KRAS* mutations in codons 12, 13, and 61 are suggested as biomarkers to predict the efficacy of anti-epidermal growth factor receptor (EGFR) treatment in colorectal cancer (Lewandowska et al., 2013). Additionally, the accumulation of several genetic alterations, such as T1796A (V599E), G1382T (R461I), T1385G (I462S), G1388A (G463E) and A1798G (K600E), are more commonly found in larger adenomas (< 1 cm across) than in smaller adenomas (Rajagopalan et al., 2002). However, a recent report about genetic progression of *BRAF*(V600E)-based tumourigenesis has provided a rationale for clinical evaluation of MEK, PI3K, and BRAF-inhibitors in *BRAF* mutant CRC (Rad et al., 2013; Popovici et al., 2012).

9. Developmental disorders: Noonan syndrome, cardio-facio-cutaneous syndrome and LEOPARD syndrome

Distinctive facial dysmorphia, reduced growth, variable cognitive deficits, cardiac defects, skeletal and ectodermal anomalies are overlapping features of three developmental disorders: NS, LEOPARD (LS), and CFC syndromes. Genetic deregulation of the *BRAF* gene (Table 1) is associated with the alteration of the RAS–MAPK signal transduction pathway, resulting in

the molecular lesions observed in NS, LS, and CFC patients (Sarkozy et al., 2009; Schulz et al., 2008; Roberts et al., 2006).

Along with *PTPN11* and *RAF1*, mutations in the BRAF gene are also associated with a disorder complex termed LEOPARD syndrome (LS-3). Mutations in these three genes have been identified in 95% of LS-affected individuals (Martinez-Quintana and Rodriguez-Gonzalez, 2012). A heterozygous *de novo* mutation (Thr241Pro) in the *BRAF* gene was found in an LS patient, as reported by Sarkozy et al. (2009). *In vitro* functional studies have identified that the *BRAF*-T241P mutation results in increased activation of the downstream MAPK pathway and is associated with developmental syndromes (RASopathies) such as NS, LS, neurofibromatosis 1 and capillary malformation-arteriovenous malformation (Tidyman and Rauen, 2009). Furthermore, Koudova et al. (2009) reported a LS patient with normal intelligence, who was characterised with a novel alteration (Leu245Phe) in the *BRAF* gene (Table 1). In addition to LS and NS, the CFC syndrome has been diagnosed by mutations in four genes—*BRAF*, *KRAS*, *MAP2K1* and *MAP2K2*—that regulate the Ras–MAPK cascade (Tidyman and Rauen, 2008; Siegel et al., 2011). The role of the *KRAS* mutations has been identified in clinically diagnosed individuals with NS and CFC syndrome (Rauen et al., 2010). Approximately 40% of the clinically diagnosed NS patients have been characterised with mutations in the *PTPN11* gene. The *RAF1* gene, which is located on chromosome 3p25.1, is also characterised by missense mutations that account for 3–17% of NS individuals (Tidyman and Rauen, 2008). However, no *PTPN11* mutation has been found in patients with CFC syndrome (Niihori et al., 2006).

10. Noonan syndrome (NS) and Noonan-like syndrome (NLS)

Noonan syndrome (OMIM: 164757) is an autosomal dominant developmental disorder characterised by facial dysmorphism including dolichocephaly, prominent forehead, hypertelorism, and low-set ears, and cardiac defects (Sarkozy et al., 2009), and is mostly associated with mutations in the *PTPN11*, *SOS1*, *BRAF* and *KRAS* genes (Tartaglia et al., 2001; Tidyman and Rauen, 2009; Gos et al., 2012; Kitsiou-Tzeli et al., 2006). However, Noonan-like syndrome (NLS) is comprised of Costello syndrome (CS; OMIM 218040), CFC syndrome (OMIM: 115150), neurofibromatosis type 1 (NF-1; OMIM: 162200), and Legius syndrome (OMIM: 611431), which are clinically associated with NS, and carries mutations in the *KRAS*, *HRAS*, *BRAF*, *MEK1*, and *MEK2* genes (Quaio et al., 2013). Due to the overlapping clinical features and alterations of the same molecular mechanisms in the RAS/MAPK pathway (RASopathies), NS and NLS have been grouped together in the neuro-cardio-facial-cutaneous syndrome family (Malaquias et al., 2012). Postnatal short stature is one of the cardinal signs of NS and NLS; however, the pathophysiological mechanism of growth aberration remains unclear.

Noonan syndrome (NS) is one of the most common dysmorphic syndromes and is described as having cardinal features such as pulmonic stenosis (PS), a distinctive dysmorphic facial appearance with hypertelorism, postnatally reduced growth, ectodermal and haematologic anomalies, variable cognitive deficits and skeletal, and congenital heart defects and hypertrophic cardiomyopathy (Tartaglia et al., 2011). Because it is a heterogeneous disorder, this Mendelian trait is casually associated with nine genes (*RAF1*, *BRAF*, *SOS1*, *KRAS*, *NRAS*, *MEK1*, *PTPN11*, *SHOC2* and *CBL*), similar to LEOPARD syndrome (Tartaglia et al., 2011). Approximately 40% of the clinically diagnosed NS individuals are characterised with *PTPN11* missense mutations (Tidyman and Rauen, 2009, 2008). Similar to *PTPN11*, missense mutations in the *SOS1* gene are the second leading cause of NS and account for ~13% of individuals (Tidyman and Rauen, 2008). Ras-GEF is responsible for stimulating the conversion of inactive Ras to its active form, and this conversion is disrupted by mutations in the *SOS1* gene, resulting in an increase in the active Ras thereby leading to an increased Ras/MAPK pathway signalling (Tidyman and Rauen, 2009). Similar to *SOS1*, missense mutations in the *RAF1* gene are responsible for 3–17% of the NS individuals (Tidyman and Rauen, 2008). However, mutations in other genes, such as *MEK1* and *BRAF*, account for $<2\%$ of the NS cases (Sarkozy et al., 2009). In exon 13 and 15 of the *BRAF* gene, Sarkozy et al. (2009) characterised Noonan syndrome-7 patients with 2 heterozygous *de novo* transversions—W531C (1593G-C) and L597V (1789C-G) respectively. Moreover, in recent reports, NS has frequently been associated with tumour and haematological malignancies (Aoki and Matsubara, 2013; Gremer et al., 2011).

11. Cardio-facio-cutaneous syndrome

Cardio-facio-cutaneous syndrome, which presents with multiple congenital anomalies and mental retardation, was first reported in 1986 (Reynolds et al., 1986). However, the phenotypic overlap between CFC syndrome and NS was emphasised by Fryer in 1991 (Fryer et al., 1991). CFC shares several phenotypic features with NS and Costello syndrome (Niihori et al., 2006). The similarities between CFC and NS can make the differentiation difficult in infants; however, the phenotypic features become more distinctive with the passage of time.

CFC is caused by mutations in four identified genes that encode proteins involved in the Ras–MAPK pathway, including the *BRAF*, *KRAS*, *MAP2K1* and *MAP2K2* genes (Tidyman and Rauen, 2008). In 2006, Niihori et al. compared the *BRAF*-positive and *KRAS*-positive individuals with CFC and identified the difference in manifestations—skin abnormalities, including ichthyosis, hyperkeratosis, and hemangioma—between the two groups (Niihori et al., 2006). However, the role of the *KRAS* gene remains unclear as *KRAS* mutations have been identified in individuals clinically diagnosed with NS as well as CFC syndrome (Rauen et al., 2010). No *PTPN11* (Niihori et al., 2006) or *HRAS* mutations have been detected in patients with CFC syndrome (Rodriguez-Viciana et al., 2006). Heterogeneous missense mutations in the *BRAF* gene are present in approximately 75% of CFC individuals with CFC-1 (OMIM: 115150) Tidyman and Rauen, 2009. The *BRAF* mutations that are associated with CFC syndrome are found in exons 6, 11, 12, 13, 14, 15 and 16. The majority of these mutations are found in exon 6 (41%) and exon 12 (21%). The most common mutation that occurs in exon 6 is a missense Q257R substitution (29% occurrence). In exons 12 and 11, the E501G (12%) and G469E (6%) missense substitutions, respectively, have been found in patients with CFC syndrome

(Rauen et al., 2010). Similar to *BRAF*, different heterozygous missense mutations in *MAP2K1* (MEK1) and *MAP2K2* (MEK2) are found in approximately 25% of CFC cases. The *MAP2K1* gene is located on chromosome 15q22.31 and encodes the mitogen-activated protein kinase 1 (MEK1), while the *MAP2K2* gene is located on chromosome 19p13.3 and encodes the protein MEK2 (Tidyman and Rauen, 2008). Both isoforms have the ability to activate *ERK1* and *ERK2* (Tidyman and Rauen, 2009).

Although, Costello, Noonan and CFC syndromes have overlapping phenotypic features, the *HRAS* gene is mutated in Costello syndrome; this is in contrast to NS, which has mutations in the *PTPN11* and *SOS1* genes, and CFC syndrome, which has mutations in the *BRAF* (75%), *MAP2K1* and *MAP2K2* (~25%), and *KRAS* (< 2%) genes. The overlap in phenotypic features may be caused by the biochemical relationship of these mutated genes in each syndrome.

12. LEOPARD syndrome

The word LEOPARD is an acronym for a combination of disorders with multiple lentigines (L), ECG abnormalities (E), ocular hypertelorism (O), pulmonary stenosis (P), abnormalities of the genitalia (A), retardation in growth (R), and deafness (D) (Gorlin et al., 1960). Although this syndrome has multiple disorders, it is not considered to have a life threatening diagnosis. Most of the people diagnosed with the aforementioned manifestations live normal lives. However, the patients with cardiac deformities have a profoundly high risk of death due to obstructive cardiomyopathy and its relevant pathologic severity towards the cardiovascular system (Jozwiak et al., 1996; Bissler et al., 2013).

LEOPARD syndrome, a rare autosomal dominant multisystem disorder, has been characterised with mutations in *PTPN11*, *RAF1*, and *BRAF* genes. Approximately 90% of all individuals with LEOPARD syndrome have a *PTPN11* mutation. In the remaining 10%, a *RAF1* mutation is most common. In a small number of cases, the underlying genetic cause is still unknown.

Sequence analysis of exons 6, 13, and 16 of the *RAF1* gene detects all reported missense mutations that are clinically associated with LS (Allanson and Roberts, 1993; Pandit et al., 2007). Additionally, sequence analysis of the *RAF1* gene has indicated that no exon or whole-gene deletions or duplications have been documented for LEOPARD syndrome, to-date (Gelb and Tartaglia, 1993). However, sequencing analysis of the *BRAF* gene has uncovered missense mutations in all the coding exons in individuals with the clinical features of LS (OMIM: 613707) Sarkozy et al., 2009; Koudova et al., 2009.

LEOPARD syndrome is quite similar to NS but occurs with a low frequency. Many features of LS show significant overlap with NS, including an autosomal dominant inheritance, similar facial dysmorphisms and similar cardiac defects. Both syndromes are caused by mutations in various genes encoding members of the RAS–MAPK signalling cascade and result in the deregulation of this pathway (Koudova et al., 2009). In LS, *PTPN11* mutations appear to inactivate the protein function, while the protein remains activated in NS. This suggests that different mechanisms underlie the two related syndromes.

13. Cancer in CFC and NS

Clinical manifestations of NS are similar to those of CFC and LEOPARD syndromes. According to recent reports, NS, LEOPARD and CFC patients have an increased risk of tumour development (Fig. 3B) (Aoki and Matsubara, 2013; Denayer et al., 2010), although no precise estimates are available yet (Hasle, 2009). Mutations in the Noonan-associated genes (*RAF1*, *KRAS*, *PTPN11* and *SOS1*) have also been found in human tumours such as colon, pancreas, thyroid, lung and melanomas (Denayer et al., 2010). The co-incidence of NS-neurofibromatosis with CFC and of rhabdomyosarcoma with NS, with somatic mutations, has been described in different reports in the literature (Hasle, 2009; Moschovi et al., 2007; Khan et al., 1995). However, there is an increased frequency of neurofibromatosis type 1 (NF-1), which results in the development of malignant schwannomas, phaeochromocytomas, and acute lymphoblastic leukaemia, in NS patients (Khan et al., 1995). First, a report about the association of Sertoli cell tumours and NS was put forth by Fryssira et al. (2008). In this report, the development of solid tumours was observed in patients diagnosed with Noonan syndrome. Pilocytic astrocytomas (Pas), granular cell tumours and Sertoli tumours in the testis are different types of tumours that have been observed in NS patients (Fryssira et al., 2008). The increased incidence of PAs (PA19, PA41 and PA42) with hyperactivation of the RAS/RAF signalling is diagnosed with clinical features of Neurofibromatosis type 1 (NF1) syndrome (Jones et al., 2008). In another published study, a very large region of copy number-LOH (CN-LOH) proximal to the location of the *NF1* gene is proposed to affect the development of multiple different tumour types, including low-grade gliomas. Different reports have identified the duplication of the 7q34 region with *HIPK2-BRAF* and *KIAA1549-BRAF* gene fusion products in low-grade paediatric astrocytomas (Sievert et al., 2009). Moreover, *FAM131B-BRAF* gene fusion, and fusion variants of *BRAF* and *RAF1* genes have been characterised in paediatric astrocytoma (Cin et al., 2011).

To explore the role of the aberrant RAS–MAPK signalling in syndromic multiple giant cell lesions (MGCL) accompanied by NS (Bertola et al., 2001; Lee et al., 2005), a cohort of 75 NS patients was identified as carrying *BRAF* mutations (Neumann et al., 2009). The occurrence of MGCL in NS and CFC patients with various underlying genetic alterations corroborates the deregulation of the RAS–MAPK pathway, which causes these patients to be predisposed to giant cell lesion (Neumann et al., 2009).

Tumour screening in CFC patients has been given little attention until the molecular analyses become available (Aoki and Matsubara, 2013). Both germline and somatic mutations in the *BRAF* and *KRAS* genes associated with CFC syndrome have been identified in hepatoblastoma (Al-Rahawan et al., 2007), non-Hodgkin lymphoma (Ohtake et al., 2011), juvenile myelomonocytic leukaemia-like (JMML) Schubbert et al., 2006, malignant melanoma (Makita et al., 2007), and cancers of the colon, thyroid and lung (Fig. 4) (Makita et al., 2007). However, JMML myeloproliferative disorders have also been characterised as containing *PTPN11* mutations, similar to NS (Hasle, 2009). Somatic *BRAF* mutations are substantially documented in haematological malignancies but with low frequency. The recent identification of

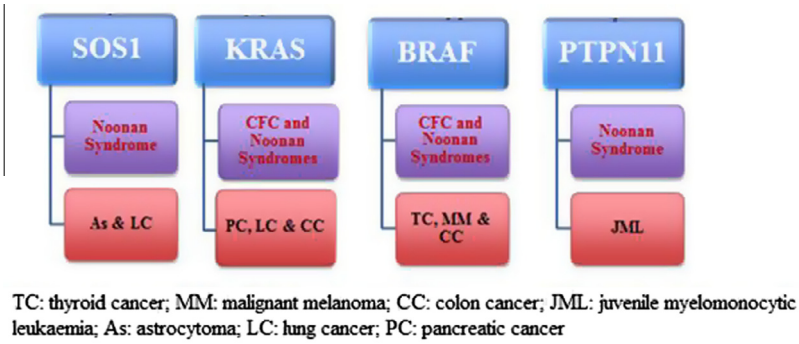

Figure 4 Genes—*KRAS*, *BRAF*, *PTPN11* and *SOS1*—characterised in both human cancers and RAS/MAPK syndromes (CFC & Noonan).

the *BRAF* mutations in Langerhans cell histiocytosis and haematological malignancies suggests that the *BRAF* gene has a role beyond solid tumours (Al-Rahawan et al., 2007).

14. Conclusion and future prospects

Mutations in *BRAF* expression have been important in understanding the roles that BRAF and other members of the RAS/RAF cascade play in disease progression, and detail mechanistic study can help to approach effective BRAF inhibitors for cancer diseases. However, growing concerns over drug resistance to molecular targeted therapies (Chakraborty et al., 2013), including BRAF and MEK inhibitors, have stimulated investigators to uncover additional molecular targets for the treatment of cancers as well as for RASopathies, in the hope of providing personalised medicine. The goal of personalised medicine is to explore different techniques associated with cancer therapy and the management of various malignancies in RASopathies. Although the regulation and inhibition of the RAS/MAPK pathway have been widely studied in cancer research, the natural history and predisposition of individuals with NS, CFC and LEOPARD syndromes, characterised as a result of disruption of the RAS/MAPK cascade, have not been understood. Additionally, the molecular phenomena of the RAS/MAPK cascade underlying overlapping features and varied degrees of penetrance in NS, CFC and LEOPARD (RAS/MAPK) syndromes are largely unknown.

In a mouse model, inhibition of mTOR, which regulates mRNA translation and ribosome synthesis, intriguingly indicated the reverse heart defects for NS multiple lentigines. Additionally, HMG-CoA reductase inhibitors and MEK inhibitors ameliorated the phenotype of the NS and NF-1 mouse models (mutations in SOS1 and RAF1) (Gelb and Tartaglia, 2011; Bauer and Stratakis, 2005; Wu et al., 2011). Such findings suggest that the complete manipulation of RAS/MAPK activity can help to correct the aberrant activation of the RAS-RAF signalling that is responsible for variable clinical phenotypes in the form of RAS–MAPK syndromes, and associated cancer risks.

Disclosure statement

The authors declare that they have no conflicts of interest.

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