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Towards precision medicine for head and neck cancer
David Vossen

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Genetic profiling of head and neck squamous cell carcinoma for (chemo)radiation response prediction

DAVID VOSSEN

Towards precision medicine for head and neck cancer

David Vossen

COLOFON

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Towards precision medicine for head and neck cancer

Genetic profiling of head and neck squamous cell carcinoma

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

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CHAPTER 1

Introduction

Precision medicine has been described as “an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person” [1]. As cancer is a disease characterized by genetic alterations, the rapid development of techniques for detecting genetic alterations and drugs targeting these alterations have greatly accelerated the implementation of precision medicine for cancer. Molecularly targeted therapies have expanded the treatment arsenal and omics measurements enable the identification of prognostic and predictive biomarkers to select subpopulations of patients that will show the best response to a given treatment. For some cancer types these developments have already reached the stage of clinical practice. For example, imatinib targets the *BCR-ABL1* fusion gene and is commonly prescribed for chronic myeloid leukemia [2,3] and MammaPrint is a commercially available microarray-based biomarker [4,5] for early stage breast cancer.

However, with regard to exploiting tumor specific genetic alterations, precision medicine for head and neck cancer (HNC) is still in the developmental stage. The research presented in this thesis is intended as a step towards precision medicine for HNC. As an introduction, here follows a description of how HNC is currently classified and treated in the clinic, of future precision medicine opportunities, and how the work presented in this thesis relates to these.

CURRENT CLINICAL PRACTICE

Anatomy, histology, epidemiology and etiology

Head and neck cancer (HNC) is an umbrella term for a variety of cancers of the upper aerodigestive tract. It is the 9th most common cancer in the Netherlands, with ~3.000 new cases annually [6]. HNCs can arise from mucosal, salivary gland, or skin tissue, and are found at various anatomic sites (Figure 1). Although HNCs can arise from different tissues, ~90% are squamous cell carcinomas (HNCCs) [7,8] arising from mucosal epithelium. HNSCCs of the oral cavity, hypopharynx, oropharynx and larynx are by far the most common HNSCCs in the Western world. Together HNSCCs from these sites account for ~80% of all HNCs in the Netherlands (Figure 2A). Therefore, we will use the abbreviation, HNSCC, to refer to tumors of these subsites in the remainder of this thesis.

Overall, HNSCC is far more common in men than in women (Table 1) [7,8]. Tobacco smoke and alcohol are the most common etiological factors of HNSCC. Long-term heavy consumption of cigarettes and alcohol greatly increases the risk of developing HNSCC [7,8]. Consequently, the typical HNSCC patient is male, above 60 years of age at diagnosis and has a history of smoking and/or alcohol dependence (Table 1) [7,8].

Persistent infection with the human papillomavirus (HPV) is a risk factor for developing oropharyngeal cancer. In comparison to HPV-negative oropharyngeal cancer, HPV-positive oropharyngeal cancer is typically found in younger and healthier patients with limited exposure to tobacco smoke and alcohol. Importantly, patients with HPV-positive and -negative HNSCC are generally viewed as distinct demographic groups (Table 1) [7,8].

Staging

Cancer staging is 'the process of determining how much cancer is in the body and where it is located' [9]. Based on this information, cancer staging systems classify tumors into groups that express disease severity [10]. Staging often is the foundation for developing a treatment plan and estimating the prognosis of a patient [11]. In this thesis, staging was performed according to the 7th edition TNM staging system published by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) [12]. The TNM staging system combines tumor location, tumor size and extent (T), regional lymph node involvement (N) and presence or absence of distant metastasis (M) into an overall disease stage (e.g. see Table 2).

The AJCC disease stages range from I-IV with increasing disease severity: I-II are considered early-stage and III-IV advanced stage. Stage IV is further divided into IVA, IVB and IVC: advanced resectable, unresectable and metastatic disease, respectively. At diagnosis, roughly 40% of patients have early-stage disease (stage I-II), 50% locoregionally advanced disease (stage III-IVB) and 10% metastatic disease (stage IVC) [7,8]. The focus of this thesis is on locoregionally advanced HNSCC.

The updated, 8th edition TNM staging system was introduced in clinical practice in 2018. Although it was not used in this thesis, it has made two noteworthy advances. Most important is the inclusion of a separate staging for HPV-positive oropharyngeal cancer, in recognition of its favorable treatment response and prognosis (Table 2). Second, is the consideration of extranodal extension (ENE) for the N stage of HPV-negative HNSCCs. In nodal metastases, ENE is the extension of cancer cells through the lymph node capsule, which is associated with poor outcome.

Treatment

Surgery, radiotherapy and chemotherapy are the main treatment modalities for HNSCC. The choice of modality, or combination of modalities, depends largely on tumor site, stage and aim of the treatment. Whereas curation is the aim for early- and locoregionally advanced stage disease, palliation is the objective for metastatic disease.

Monotherapy consisting of either surgery or radiotherapy, depending on tumor site, is the preferred treatment for early-stage disease. In contrast, multimodal treatment is used for locoregionally advanced disease. Surgery followed by postoperative (chemo-)radiotherapy is the standard of care for advanced stage tumors of the oral cavity and T4 laryngeal tumors. Cisplatin-based concurrent chemo-radiotherapy, with surgery reserved for salvage treatment, is the treatment of choice for tumors of the larynx, oropharynx and hypopharynx, with the exception of T4 laryngeal tumors [13,14]. For distant disease, a single chemotherapeutic or multiple chemotherapeutics are used for palliation, depending on the patient's performance status and comorbidities.

The only targeted therapy approved for HNSCC is cetuximab, a monoclonal antibody to EGFR. Currently, cetuximab is used as a replacement of cisplatin for patients that cannot tolerate cisplatin due to poor performance status or comorbities, during chemo-radiotherapy and palliative chemotherapy. Cetuximab efficacy in patients with HPV-positive oropharyngeal cancer is currently questioned [15].

Prognosis

Stage at diagnosis is one of the strongest prognostic factors for HNSCC patients [16,17]. Consequently, the overall survival per tumor site is closely related to the ratio of early to advanced stage tumors at diagnosis (Figure 2B) [17]. Accordingly, tumors of the larynx have the best prognosis, followed by those of the oral cavity, oropharynx and hypopharynx (Figure 2C). This thesis focuses on locoregionally advanced HNSCC treated with platinum-based chemo-radiotherapy. For this group, the overall 5-year survival is 30-45% and the 5-year cancer-related death rate ~50% [18–21].

PRECISION MEDICINE

The poor prognosis of locoregionally advanced HNSCC illustrates that the current diagnostic and therapeutic approaches are insufficient. Although the TNM staging system classifies tumors into disease stages with distinct prognoses [11], it explains only 10-30% of the variance in survival rates [10,22–24]. In practice this means that patients with the same disease stage can have a markedly different outcome. This implies that some patients are treated adequately, but others are over- or undertreated. Therefore, a more precise treatment approach is needed, one that takes more information into account than just patient factors, TNM stage, and tumor site. As cancer is largely a genetic disease, variation in the genetic makeup of tumors is thought to explain part of the clinical variance uncaptured by the TNM staging system. As a result, there is a great scientific effort ongoing to discover prognostic and predictive biomarkers based on genetic information. In addition, drugs targeting specific genetic alterations are under investigation.

Opportunities for biomarkers and targeted drugs in HSNCC treatment both arise from the genomic characteristics of HNSCC. Therefore, we first provide an overview of these characteristics and then a description of various treatment and biomarker opportunities arising from it. In addition, promising non-genetic opportunities are described.

Genomic characteristics of HNSCC

Study of the genomic alterations in cancer was profoundly changed and accelerated by the introduction of commercial DNA sequencing machines in the mid 2000s [25]. These sequencers allowed affordable and fast sequencing of (parts of) genomes, commonly referred to as 'next-generation sequencing' (NGS). Soon after the introduction of NGS, large-scale projects were undertaken to sequence and map the genomic alterations of cancer. The Cancer Genome Atlas (TCGA) [26] is probably the best known example of such projects. TCGA performed molecular characterization at the DNA, RNA, protein, and epigenetic levels [26]. Preceded by many smaller HNSCC sequencing studies [27–35], TCGA published the most comprehensive molecular characterization of HNSCC to date in 2015 [36]. This study identified novel frequently altered genes and confirmed the molecular differences between HPV-positive and -negative HNSCC and the most frequently altered pathways in HNSCC (Figure 4). In summary, roughly a dozen genes are frequently mutated in HNSCC (Table 3) and a much larger number of genes is mutated infrequently.

Treatment

In the future, the therapeutic arsenal against HNSCC might include targeted inhibitors, immunotherapy, poly ADP-ribose polymerase (PARP) inhibitors, and hypoxia modifiers. The genomic characteristics of HNSCC point to opportunities for targeted inhibitors [37,38]. Driver mutations in oncogenes can cause a dependency of cancer cells on the affected pathways [39]. Drugs that selectively inhibit these activated oncogenes are a key advancement in precision oncology [40]. Only three of the frequently mutated (candidate) driver genes in HNSCC are oncogenes: *EGFR*, *PIK3CA*, and *CCND1* (Table 3) [41]. Amplification of *EGFR* is already targeted in routine clinical practice with cetuximab. The other two frequently mutated oncogenes, *PIK3CA* and *CCND1*, are promising candidates for targeted therapy [37,38,42–44].

In HNSCC, frequent gain-of-function mutations have been found in *PIK3CA* and other PI3K/AKT/mTOR pathway members [31,36]. Constitutive activation of the PI3K/AKT/mTOR pathway by gain-of-function mutations makes it an important oncogenic signaling pathway. Therefore, multiple PI3K/AKT/mTOR pathway inhibitors have been developed. In preclinical studies, some of these inhibitors have demonstrated efficacy in HNSCC [42–44]. However, the clinical trials that are currently investigating these drugs for locally advanced, recurrent and metastatic HNSCC have not found significant efficacy yet [38,43,44]. This may be due to several factors, notably

patient selection and acquired resistance. The current trials have not required PI3K/AKT/mTOR pathway mutations for enrollment and most have not stratified patients according to these mutations either [38,44]. PI3K/AKT/mTOR pathway mutation status or predictive biomarkers may be essential to select patients that can benefit from targeted treatment, and hence demonstrate efficacy of these drugs. On the other hand, acquired resistance to PI3K/AKT/mTOR pathway inhibitors may limit their efficacy [40]. Investigation of resistance mechanisms might point to solutions, as is illustrated by advancements in the treatment of other cancer types such as melanoma. *BRAF* is frequently mutated in melanoma, but melanomas quickly develop resistance against monotherapy with BRAF inhibitors. However, survival of melanoma patients can be significantly prolonged by combined inhibition of the MAPK pathway members BRAF and MEK [45]. Furthermore, a recent study showed that the melanoma cells that do eventually develop resistance against this combination therapy acquire properties that can again be therapeutically exploited [46]. The PI3K/AKT/mTOR pathway is thus an attractive therapeutic target in HNSCC, but additional research may be required to translate this into improved patient survival.

CCND1 amplification and *CDKN2A* inactivation, either via deletion or somatic point mutation, are frequent in HNSCC (Table 3). The protein product of *CCND1* (cyclin D1) binds to either CDK4 or CDK6. The resulting complex contributes to cell cycle progression and is inhibited by the protein product of *CDKN2A* (p16). Either *CCND1* gain or *CDKN2A* loss could thus promote cell cycle progression. As these mutations are both frequent in cancer, CDK4/6 inhibitors have been developed. Such inhibitors have demonstrated value in breast cancer [47] and are currently being evaluated for HNSCC in clinical trials [37,38,48]. Like with other targeted therapies, careful patient selection and predictive biomarkers will be crucial to effectively employ CDK4/6 inhibitors.

There is currently great enthusiasm surrounding immunotherapy and the combination of immunotherapy and radiotherapy for cancer treatment [49,50]. HNSCC is a promising candidate disease for immunotherapy, as it employs various mechanisms of immune suppression [37,38,51] and is frequently treated with radiotherapy. Indeed, the immune checkpoint inhibitors nivolumab and pembrolizumab have been found to benefit patients with HNSCC that is recurrent or metastatic after platinum-based chemotherapy [52,53]. Consequently, both drugs have received FDA approval for second-line treatment of this patient population. Several clinical trials are further evaluating the usefulness of immune checkpoint inhibitors for HNSCC [37,38,51,54]. These and other future trials will elucidate their use as first- and second-line treatment, for locally advanced and recurrent or metastatic HNSCC, and in combination with various standard of care treatment modalities.

PARP inhibitors are another class of drugs under investigation for use against HNSCC [42]. PARP inhibitors can be used either as monotherapy or as radiosensitizers. PARP inhibitors are

particularly effective in homologous recombination (HR) deficient cells (for more see Chapter 6), but HR proficient cancer cells may also be targeted via proliferation-dependent mechanisms when combined with radiation or chemotherapy [55,56]. Indeed, a preclinical study by our group found that the PARP inhibitor, olaparib, radiosensitized HR proficient cells, but to a lesser extent than HR deficient cells [57]. Our group and others have reported similar findings in HNSCC cells [57,58]. PARP inhibitors might thus be used against HNSCC in the future, and clinical trials are underway [59]. Yet, predictive biomarkers are crucial to maximize the chance of success of these trials.

Hypoxic modifiers are another class of drugs that are being studied for radiosensitization in HNSCC [60]. As oxygen is important for the biological effectiveness of radiation, low oxygen (hypoxia) reduces the efficacy of radiotherapy and consequently lowers tumor control and survival [61]. Hypoxic areas are a common feature of solid tumors and various strategies and drugs to target hypoxia have therefore been developed [60,62,63]. A meta-analysis found that adding hypoxic modification to radiotherapy improved locoregional control, disease specific survival and overall survival in HNSCC [60]. As hypoxic modification is aimed at hypoxic tumors, multiple hypoxia imaging [61] and gene expression signatures [64–67] are under investigation to identify these tumors.

In addition to exploring novel treatment opportunities, research has also identified factors related to the current treatment that can be optimized. For example, it has been shown across a wide range of procedures and conditions that there is a positive correlation between the number of times that a physician performs a procedure and patient outcome [68,69]. Examples of treatment-related factors that have been studied in HNSCC specifically include waiting time until treatment [70,71], cumulative cisplatin dose [72], cisplatin dosing schedule [73], order of treatment [18,19] and radiotherapy fractionation schemes [21].

In short, there are various classes of drugs that are being evaluated for HNSCC treatment. Each of these novel drugs requires tumor cells to have particular characteristics in order to be effective. Therefore, predictive biomarkers and careful patient enrolment are required to demonstrate efficacy of these drugs in clinical trials. Once approved, such biomarkers will also be crucial to cost-effectively apply these drugs in the clinic.

Biomarkers

Biomarkers can be roughly categorized as prognostic or predictive. Prognostic biomarkers inform about disease outcome irrespective of the treatment administered. In contrast, predictive biomarkers inform about the treatment effect – which differs between patients that are positive and negative for the biomarker [74]. Currently, HPV status determined by immunohistochemistry

and polymerase chain reaction (PCR) is the only prognostic biomarker for HNSCC that has advanced into the clinic. HPV-positive oropharyngeal cancer is a subgroup of oropharyngeal cancer with favorable prognosis [75–77] with a distinct tumor biology [29,35]. Furthermore, p16 overexpression detected by immunohistochemistry is an affordable and robust biomarker for HPV-positivity [11]. Consequently, HPV status is now considered in the prognostication of HNSCC via separate staging tables for p16-positive and -negative oropharyngeal cancer (Table 2). Although HPV status is an established prognostic biomarker, it still has no influence on treatment selection. However, studies aiming to reduce treatment-related toxicities in HPV-positive HNSCC are investigating treatment de-escalation for these patients [78].

Other than HPV status, there is a lack of HNSCC biomarkers. Even for the targeted EGFR inhibitor, cetuximab, no predictive biomarker exists despite advancing insight [79]. The focus of this thesis is on moving towards precision medicine for locoregionally advanced HNSCC treated with platinum-based chemo-radiotherapy. The high 5-year locoregional failure rate of chemo-radiotherapy treated tumors (~50%) [19] suggests that a considerable proportion is unresponsive to this treatment. If unresponsive tumors could be identified beforehand, patients could be spared chemo-radiotherapy induced toxicities by receiving surgery upfront, which they would otherwise have received as salvage treatment. Alternatively, these tumors might benefit from radio-sensitizing therapies such as immunotherapy, PARP inhibitors and hypoxia modifiers, or other molecularly targeted therapies. Furthermore, the addition of chemo- to radiotherapy improves the locoregional failure rate with ~10% [19], suggesting that only a proportion of patients benefit. Ideally only these patients would receive chemotherapy, as the addition of chemo- to radiotherapy is associated with increased toxicities [19]. Finally, a proportion of unresponsive tumors might be particularly aggressive and difficult to control irrespective of treatment modality. Genomic characterization of these tumors might point to novel treatment opportunities. Taken together, these examples illustrate the need for predictive biomarkers for radiotherapy and chemotherapy and general prognostic markers in HNSCC. Such biomarkers are necessities for the realization of precision medicine using the currently available treatments.

Therefore, research continues to search for novel biomarkers for HNSCC. Identifying prognostic factors typically precedes biomarker development. These factors are often patient or tumor characteristics. Examples of patient characteristics that have prognostic value in HNSCC are: smoking [80], age [16,17,80,81], obesity [81], comorbidities [16], and socioeconomic status [17]. TNM stage is the most obvious example of a prognostic tumor characteristic. However, technological advancements have allowed researchers to measure an increasing number of tumor features. Our group has made considerable contributions to the development of HNSCC biomarkers using microarrays. We have developed biomarkers of radiosensitivity based on cell line [82,83] and patient [84] derived mRNA and microRNA expression profiles. Furthermore, we

have developed prognostic biomarkers for chemo-radiated HNSCC based on patient material using immunohistochemistry [85], comparative genomic hybridization [86], and mRNA expression [87] data.

Examples of other tumor-related features studied in HNSCC are tumor volume calculated from CT or MRI scans [88,89], features digitally extracted from CT or MRI scans (‘radiomics’) [90,91], and hypoxia levels using medical imaging [61] and gene expression profiling [64–67]. It is also common to obtain molecular data from tumors and use these data to train predictors of (factors related to) patient outcome. Examples of molecular data that have been studied in HNSCC include gene expression levels using microarrays and RNA sequencing [36,82–84,87], copy number aberrations using array comparative genomic hybridization and SNP arrays [36,86], and somatic point mutations using DNA sequencing [27,28,36]. Such measurement techniques have succeeded each other so quickly that some have already become obsolete. However, none have yet been integrated in routine clinical practice.

Rather than being an exhaustive list of prognostic factors, these examples illustrate the diversity of prognostic factors in HNSCC. There is also much variability among these factors in terms of cost and accessibility to the treating physician and the patient. For example, whereas obesity can be easily assessed by any physician, measuring gene expression profiles requires equipment and expertise that are unavailable in most hospitals. Ultimately, cost-benefit analyses will be required for clinical application and a variety of features will likely be integrated into mathematical models [92,93].

Genomic instability and the other hallmarks of cancer

Cancer is a disease characterized by genetic alterations affecting many cellular pathways and processes. In 2000, Hanahan and Weinberg proposed that the complexities of cancer can be reduced to a small number of underlying principles: the hallmarks of cancer [94]. In their original article, they presented six hallmarks that normal cells acquire during their transformation into cancer cells (Figure 3A). These hallmarks are acquired through somatic alterations in a number of key genes [95,96]. Somatic alterations that provide a selective growth benefit to the cell and drive cancer progression are known as drivers [95]. As somatic alterations occur largely at random throughout the genome, most do not provide a selective growth advantage to the cell. Consequently, in order to acquire the drivers necessary for carcinogenesis, cells pick up thousands of alterations that do not cause a selective growth advantage, called passengers [95,97,98]. Such high alteration rates do not occur in normal cells, as these have mechanisms to efficiently repair and prevent propagation of genetic alterations. Therefore, in order to transform to cancer cells, normal cells must overcome these protective measures to acquire sufficient drivers to become malignant [97,98]. One way to accomplish this is by developing genomic

instability. Because genomic instability is paramount to carcinogenesis in many cancers, it has been incorporated as an enabling characteristic among the hallmarks of cancer (Figure 3B) [94,99,100].

Cellular processes involved in the maintenance of genomic stability are also of central importance in the treatment of HNSCC, as radiotherapy and cisplatin both function by generating DNA damage. Radiotherapy causes DNA damage either directly by interacting with DNA or indirectly via the formation of free radicals, that in turn damage DNA. Direct and indirect DNA damage cause various DNA lesions, including DNA base alterations, single-strand breaks and double-strand breaks [101,102]. DNA base alterations and single-strand breaks are far more common than double-strand breaks, but can be repaired rapidly [102]. In contrast, fewer double-strand breaks are induced, but they are considered the most detrimental and, unrepaired, are primarily responsible for radiation induced cell death.

Cisplatin functions both as a cytotoxic agent and as a radiosensitizer. Its primary mechanism of action is its interaction with DNA, resulting in the formation inter- and intra-strand crosslinks. These crosslinks hinder DNA replication, which can lead to cell cycle arrest and apoptosis [103]. In addition, cisplatin has a synergistic interaction with radiotherapy and thus radiosensitizes cells via various mechanisms [102,104]. These mechanisms may include increasing the formation of single- and double-strand breaks created by radiation induced free radicals [105] and hindering single- and double-strand break repair [106,107].

Cells have various pathways to repair DNA damage inflicted by endogenous and exogenous factors, such as radiation and cisplatin. The Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) pathways can both repair double-strand breaks. Although repair by HR is more accurate than NHEJ, cells also use NHEJ to repair double-strand breaks induced by radiotherapy. DSB repair pathway use depends on various factors, including cell type, status, and cycle phase [101]. Cisplatin induced crosslinks can be repaired by the combined activity of the Fanconi Anemia (FA) and HR pathways [108]. Furthermore, the FA/HR pathways are of great importance for the maintenance of genomic stability [109]. Consequently, defects in the FA/HR pathways can result in genomic instability and increased sensitivity to DNA crosslinking agents such as cisplatin. Such defects are therefore interesting biomarker candidates that can be investigated with DNA sequencing technologies.

Challenges in head and neck cancer genome research

After the genomic alterations of HNSCC were established, research focus shifted towards using these alterations as biomarkers [110–117]. This endeavor is complicated by HNSCC's clinical heterogeneity and relatively low incidence. Due to clinical heterogeneity, many of the published

cohorts comprise tumors from various disease stages and anatomic sites, that were treated with different combinations of multiple treatment modalities. In such studies, it is uncertain to which tumors the identified biomarkers apply. A solution is to investigate cohorts that are homogenous in terms of (sub)site, disease stage, and treatment.

Such homogenous cohorts are typically small (~50-150 patients) owing to HNSCC's heterogeneity and relatively low incidence. From a statistical perspective, a sample size that is small relative to the number of possible predictors increases the risk of overfitting. This is a concern in cancer biomarker research, given the abundance of genomic alterations in cancer genomes, that each are possible predictors.

However, the use of statistical guidelines and the genomic characteristics of HNSCC greatly reduce the number of genes that are relevant to biomarker research. In order to accurately evaluate the prognostic value of a predictor (i.e., a genomic alteration), the number of events (e.g., death, disease progression, local recurrence) per predictor should be at least 10 [118,119]. Only ~10-15 genes are altered frequently enough in HNSCC to meet this criterion in small cohorts, after correcting for background mutation rates [36,120]. Focusing on these frequently altered genes is thus a valid strategy for data-driven HNSCC biomarker research. Another strategy is investigating the prognostic value of pathways as a whole, instead of single genes (e.g., PI3K/AKT/mTOR pathway mutated versus wildtype). Alternatively, all genetic alterations in a tumor can be decomposed into mutational signatures [121,122] or combined into a numeric predictor [123,124]. Finally, hypothesis-driven biomarker research, i.e., investigating the prognostic value of preselected (sets of) gene(s) or pathway(s), by definition reduces the number of predictors. It therefore is another useful approach to develop biomarkers in small, homogenous HNSCC cohorts based on genomics data.

THESIS OUTLINE

The poor prognosis of advanced HNSCC underscores the need for precision medicine. Biomarkers are a logical first step towards this goal, within the confines of the currently available treatment modalities. As genomic alterations lie at the heart of carcinogenesis and tumor behavior, they are interesting biomarker candidates. A subset of a tumor's genomic alterations can be detected with NGS. Based on these principles and the current state of cancer research we hypothesized that NGS data can contribute to the prognostication of HNSCC. Using NGS, this thesis therefore aims to investigate the prognostic or predictive value of genomic alterations in HNSCC.

As the FA/HR pathway maintains genomic stability and repairs DNA crosslinks, FA/HR pathway deficiency might be a prognostic or predictive biomarker in HNSCC. In Chapter 2 we determine

the functionality of the FA/HR pathway in a panel of 29 HNSCC cell lines using multiple functional assays. After finding FA/HR defects in a subset of this panel, we use variants in the FA/HR genes as markers for these functional defects. Finally, we investigate the prognostic and predictive value of these variants in a cohort of 77 advanced stage HNSCC patients that received cisplatin-based chemo-radiotherapy.

Chapter 3 elaborates on the variant selection procedure that was applied in Chapter 2. Loss of the wildtype allele (or loss of heterozygosity – LOH) of FA/HR genes is a way to lose FA/HR pathway activity and we therefore employed this characteristic as a variant selection criterion. In addition, we employed variant minor allele frequency as a selection criterion. In breast and ovarian cancer research, FA/HR variants are often selected based on known involvement in these cancer types. Involvement is deduced from the high prevalence of these cancers in carriers of these variants. The high prevalence of breast and ovarian cancer in carriers of germline BRCA1 and 2 mutations suggests an interaction with reproductive hormones. In contrast, carcinogens from tobacco smoke and alcohol induce the DNA damage that possibly puts stress on the FA/HR pathway in HNSCC. A different subset of FA/HR variants might therefore be pathogenic in HNSCC than in breast and ovarian cancer. Discovering these variants therefore requires a different variant selection approach than one that is guided by breast or ovarian cancer incidence.

Chapter 4 shows a comparison between genetic profiles of oral squamous cell carcinomas (OSCC) with laryngeal and pharyngeal SCCs (LSCC and PSCC, together L/P-SCC). Studies have found worse chemo-radiotherapy response rates in OSCC than in L/P-SCC. Although this difference might partially be attributable to clinical characteristics, it also suggests that these tumors might differ in biological features relevant to chemo-radiotherapy response. We therefore compared the total and driver gene-specific rates of somatic alterations between OSCC and L/P-SCC. In addition, we compared the rates HR defects between OSCC and L/P-SCC, using multiple HR associated mutational and genomic scar signatures.

In Chapter 5 we investigate the prognostic value of driver genes and markers of genomic instability in a cohort of 77 advanced stage HNSCC patients that received cisplatin-based chemo-radiotherapy.

Finally, in Chapter 6 the results of chapters 2-5 are discussed. These results are placed in the context of contemporary HNSCC research, cancer research in general and possible future directions.

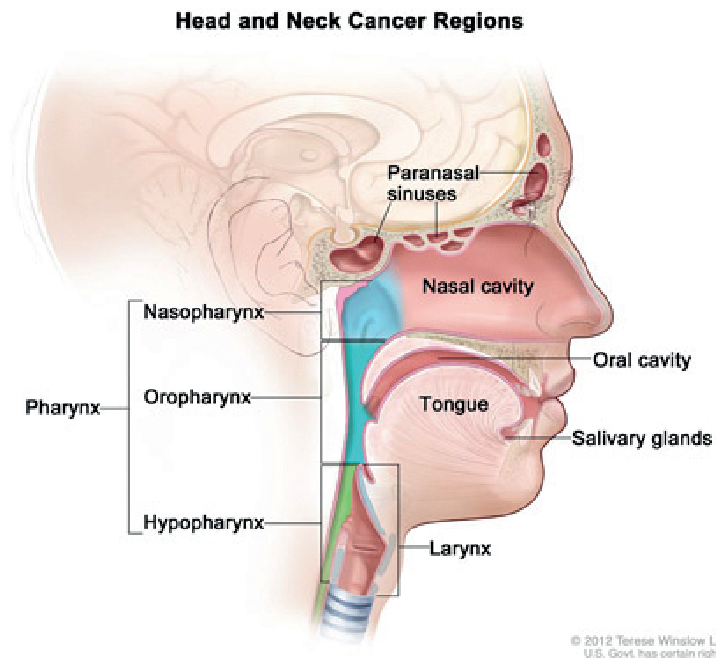
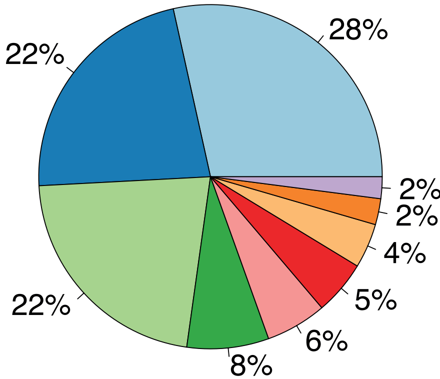


Figure 1. Head and neck cancer sites.

Illustrates location of paranasal sinuses, nasal cavity, oral cavity, tongue, salivary glands, larynx, and pharynx (including the nasopharynx, oropharynx, and hypopharynx).
Figure and figure legend copied and adapted from the NIH National Cancer Institute website [125].

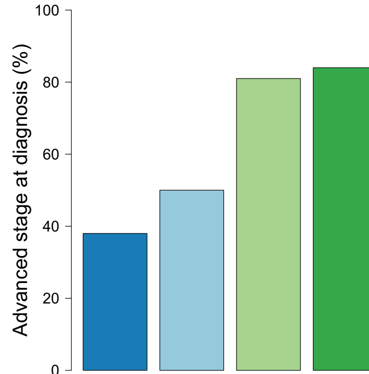
A

Relative incidence HNCs
The Netherlands, 2016



Oral cavity (n = 850)
Larynx (n = 667)
Oropharynx (n = 655)
Hypopharynx (n = 230)
Salivary glands (n = 171)
Lip (n = 152)
Nasal cavity and paranasal sinuses (n = 125)
Unknown primary site (n = 73)
Nasopharynx (n = 61)

B



C

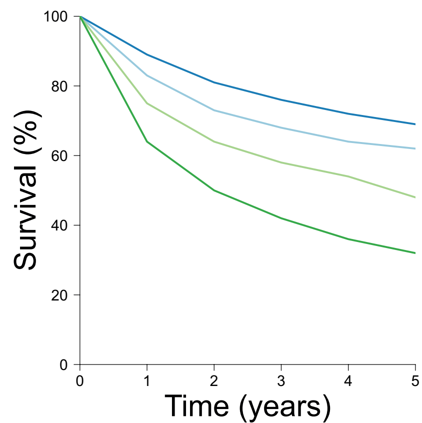


Figure 2. Head and Neck Cancer (HNC) statistics.

(A) Relative and absolute (in brackets) incidence of HNCs in the Netherlands, 2016 [6].
Cancers not shown because relative incidence below 1%:
- Melanoma of the upper aerodigestive tract (n = 10)
- Oral cavity and pharynx, not otherwise specified (n = 1)

(B) Percentage of tumors diagnosed at an advanced stage in Denmark between 1992-2008, per site [17].
(C) Overall survival per tumor site in the Netherlands between 2011-2015, relative to the general population [6].

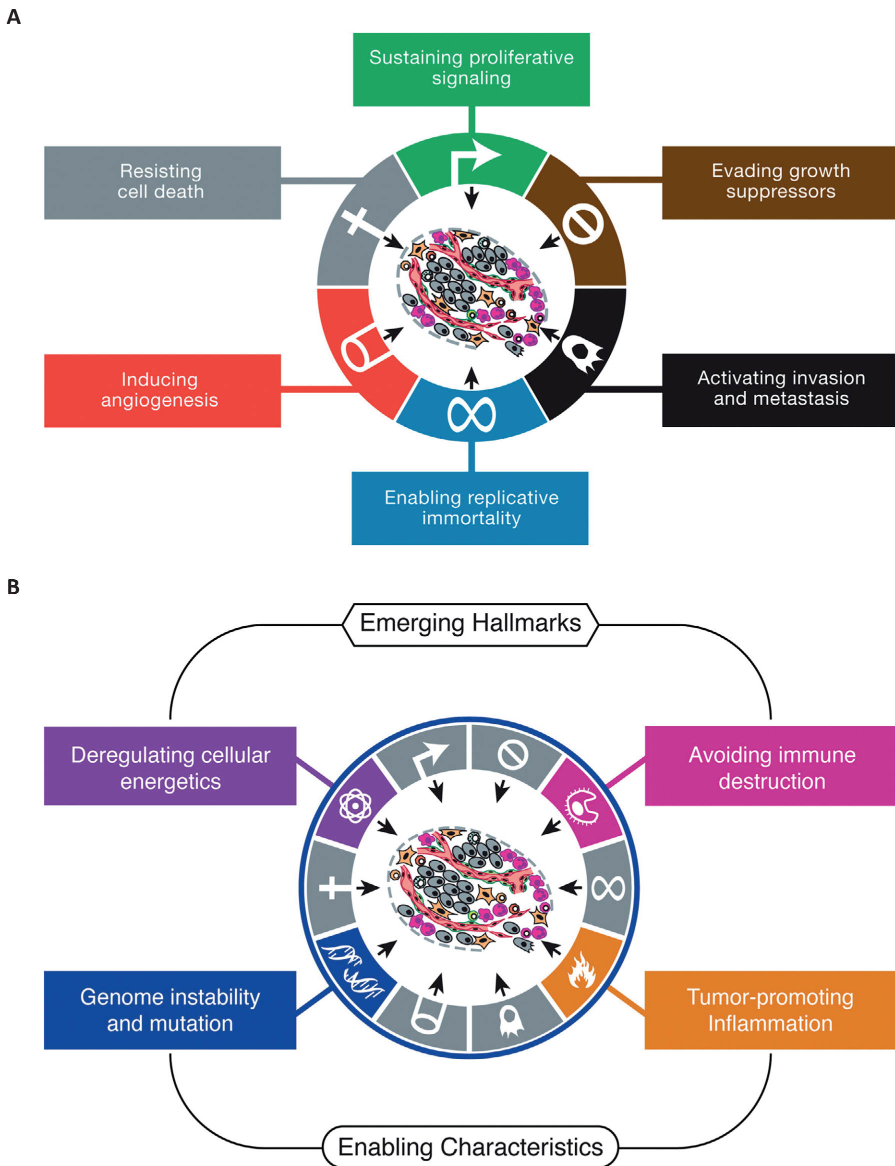


Figure 3. Hallmarks of cancer.

(A) The six core hallmarks, acquired capabilities of cancer, proposed in the original article of Hanahan and Weinberg [94]. **(B)** The updated hallmarks of cancer, including four new hallmarks. Two emerging hallmarks involved in the pathogenesis of cancer were added. In addition two enabling characteristics that facilitate the acquisition of the core and enabling hallmarks were included [100].

Figures copied from Hanahan and Weinberg [100].

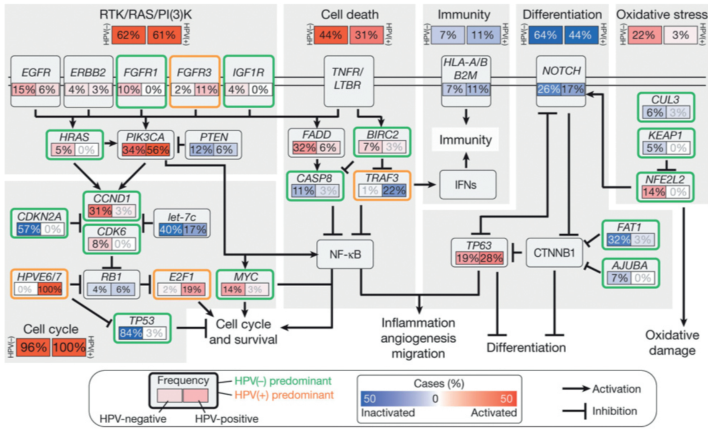


Figure 4. Deregulation of signaling pathways and transcription factors in HNSCC | The frequency (%) of genetic alterations for HPV(−) and HPV(+) tumors are shown separately within sub-panels and highlighted. Pathway alterations include homozygous deletions, focal amplifications and somatic mutations. Activated and inactivated pathways/genes, and activating or inhibitory symbols are based on predicted effects of genome alterations and/or pathway functions.

Figure and figure legend copied and adapted from TCGA [36].

Table 1. Clinical phenotype of HPV-positive and –negative HNSCC

Table copied from Marur et al. [8].

Variable	HPV positive	HPV negative
Race	White > black	White > black
Age (y)	40-60	>60
M:F	8:1	3:1
Socioeconomic status	Higher	Low-middle
Smoking/alcohol dependence	Never or minimal	Significant
Marijuana use	Strong association	Unknown
Early sexual experience	Strong association	Unknown
Multiple sexual partners	Strong association	Unknown
Tumor (T) stage	Early T stage	More advanced T stage
Nodal (N) stage	Advanced N stage	Early N stage
Distant metastasis		
DCR (%)	70-90	70-90
Second primary rate (%)	11	4.6
Overall response to treatment		
2-Year OS (%)	94 (95% CI, 87-100)	58 (95% CI, 49-74)
2-Year PFS (%)	85 (95% CI, 74-99)	53 (95% CI, 36-67)

DCR = distant metastases control rate; F = female; HNSCC = head and neck squamous cell carcinoma; HPV = human papillomavirus; M = male; OS = overall survival; PFS = progression-free survival.

Table 2. Separate staging tables for HPV-positive and -negative oropharyngeal cancer.

(A) Anatomic Stage and Prognostic Groups for *Clinical* TNM Grouping of Human Papillomavirus-Associated (p16-Positive) Oropharyngeal Cancer, 8th Edition Staging Manual. Any M1 is stage IV.
(B) Anatomic Stage and Prognostic Groups for *Clinical* and *Pathologic* TNM Grouping of Non-Human Papillomavirus-Associated (p16-Negative) Oropharyngeal Cancer, 8th Edition Staging Manual. Any M1 is stage IV.
Tables copied from Lydiatt et al. [11].

A.

	N CATEGORY			
T CATEGORY	**N0**	**N1**	**N2**	**N3**
T0	NA	I	II	III
T1	I	I	II	III
T2	I	I	II	III
T3	II	II	II	III
T4	III	III	III	III

B.

	N CATEGORY			
T CATEGORY	**N0**	**N1**	**N2a,b,c**	**N3a,b**
T1	I	III	IVA	IVB
T2	II	III	IVA	IVB
T3	III	III	IVA	IVB
T4a	IVA	IVA	IVA	IVB
T4b	IVB	IVB	IVB	IVB

Table 3. Genes with frequent and highly significant somatic genetic changes in HPV-negative HNSCC.

Table copied and modified from Leemans et al. [41].

Cellular process	Gene	Type of gene	Mutation frequency (%)	CNA frequency (%)
Cell cycle	*CDKN2A*	Tumour suppressor	22	32
	TP53	Tumour suppressor	72	1.4
	CCND1	Oncogene	0.6	25
Growth signals	*EGFR*	Oncogene	4	11
Survival	*PIK3CA*	Oncogene	18	21
	PTEN	Tumour suppressor	3	4
WNT signalling	*FAT1*	Tumour suppressor	23	8
	AJUBA	Tumour suppressor	7*	1
	NOTCH1	Tumour suppressor	18	4
Epigenetic regulation	*KMT2D*	Tumour suppressor	16	0.4
	NSD1	Tumour suppressor	12*	0.8

*Putative passenger mutation that requires further functional studies.

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CHAPTER 2

Fanconi anemia and homologous recombination gene variants are associated with functional DNA repair defects in vitro and poor outcome in patients with advanced head and neck squamous cell carcinoma

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ABSTRACT

Mutations in Fanconi Anemia or Homologous Recombination (FA/HR) genes can cause DNA repair defects and could therefore impact cancer treatment response and patient outcome. Their functional impact and clinical relevance in head and neck squamous cell carcinoma (HNSCC) is unknown. We therefore questioned whether functional FA/HR defects occurred in HNSCC and whether they are associated with FA/HR variants. We assayed a panel of 29 patient-derived HNSCC cell lines and found that a considerable fraction is hypersensitive to the crosslinker Mitomycin C and PARP inhibitors, a functional measure of FA/HR defects. DNA sequencing showed that these hypersensitivities are associated with the presence of bi-allelic rare germline and somatic FA/HR gene variants. We next questioned whether such variants are associated with prognosis and treatment response in HNSCC patients. DNA sequencing of 77 advanced stage HNSCC tumors revealed a 19% incidence of such variants. Importantly, these variants were associated with a poor prognosis (p = 0.027; HR = 2.6, 1.1-6.0) but favorable response to high cumulative cisplatin dose. We show how an integrated *in vitro* functional repair and genomic analysis can improve the prognostic value of genetic biomarkers. We conclude that repair defects are marked and frequent in HNSCC and are associated with clinical outcome.

INTRODUCTION

Chromosomal stability is governed by DNA damage response and repair processes such as the homologous recombination (HR) and Fanconi Anemia (FA) pathways. The FA-pathway is essential for the repair of DNA interstrand crosslinks and together with elements of the homologous recombination (HR) repair pathway they also strongly determine cellular survival upon exposure to crosslinking agents [1]. Aberrations in the FA/HR-pathway have been reported in multiple cancer types and their therapeutic exploitation has been described [2,3]. The breast cancer susceptibility genes *BRCA1* and *BRCA2* (*BRCA1/2*), well-known members of the FA/HR-pathway, have a well-described role in hereditary breast and ovarian cancer. Recent DNA sequencing studies highlight the high occurrence of DNA repair gene aberrations; however, the assessment of a functional impact lags behind and their clinical relevance remains poorly defined. Notably, the underlying DNA repair defects in *BRCA1/2* mutated breast and ovarian tumors can be exploited with PARP inhibitors [4–6] further stressing the importance of functional DNA repair defect studies.

Fanconi anemia patients suffer from a condition caused by germline mutations in the Fanconi anemia (FA) genes and have an increased susceptibility to cancer. Head and neck squamous cell carcinoma (HNSCC) is the most common solid cancer in these patients, with a 700-fold increased risk [7,8]. Sporadic HNSCC is the sixth most common cancer worldwide and its incidence is strongly associated with alcohol consumption, smoking and HPV infection [9,10]. A considerable proportion of patients is diagnosed at an advanced stage, at which patients are often treated with surgery or a combination of radiotherapy and cisplatin. This combination is effective, although not all patients benefit and less than half of the patients will be cured [11]. In addition, many suffer severe side effects without possibly benefiting from the treatment. New treatment decision aids and alternative therapeutic approaches are therefore urgently needed [12–14].

The strong impact of smoking and alcohol in the development of HNSCC, both likely based on the DNA crosslinking nature of these mutagens [15,16], suggests a protective role of the FA/HR repair pathway. Meta-analysis has shown the benefit of the addition of crosslinking agents to radiotherapy to improve outcome in HNSCC [11] and may further indicate tumor DNA repair defects to be involved in crosslinker sensitivity. Together these data point to a role of crosslink repair defects, particularly those of the FA/HR pathway, in the etiology and treatment of HNSCC.

In sporadic HNSCC, downregulation of FA gene expression [17] and frequent *FANCF* silencing by methylation was found [18]. Furthermore, copy number alterations [19] and somatic mutations of individual FA genes have been described in HNSCC [20,21]. A recent study found FA gene variants in HNSCC cell lines that were responsive to a chromosomal breakage assay [22]. Comprehensive

genomic analysis of the FA/HR pathway are rare and it is unknown whether these alterations compromise cellular crosslink repair activity, as functional analyses are lacking [23]. Importantly however, the clinical relevance of functional or genetic FA/HR tumor defects has not been elucidated.

In this study we therefore investigate the incidence and properties of functional DNA repair defects in HNSCC by applying multiple functional assays to a large HNSCC cell line panel. We then integrate data from these functional assays and DNA sequencing to improve the selection of functionally relevant genetic alterations. Finally, we probe the association of such FA/HR aberrations with clinical outcome in a well-defined homogenous HNSCC patient cohort (n = 77) treated with radiotherapy and cisplatin to test their prognostic value.

RESULTS

Hypersensitivity to the DNA crosslinking agent mitomycin C reveals functional crosslink repair defects in HNSCC

Hypersensitivity to the crosslinking agent mitomycin C (MMC) and a strong G2 cell cycle block in response to MMC are hallmarks of FA-pathway disruption [24,25]. To test whether sporadic HNSCCs have such DNA repair defects, we treated 29 HNSCC cell lines with MMC and assessed their survival in long term growth assays. The HNSCC cell lines showed a broad spectrum of sensitivities to MMC (Figure 1A) with IC_{50} values ranging over 50-fold from 5-250nM (Figure 1B, Supplementary Table 1). MMC-hypersensitivity, in particular if as pronounced as in the FA-patient derived cells, strongly suggests a functional crosslink repair defect in a significant proportion of the cell lines.

To confirm this FA-like phenotype additional functional endpoints were analyzed. We first examined cell cycle progression after treatment with MMC (Figure 1C, Supplementary Figure 1, Supplementary Table 1). Exponentially growing cells were treated with MMC and analyzed by flow-cytometry for cell cycle phase distribution 48hrs after treatment. Both FA-positive controls showed a strong G2-block. Consistent with the prior analysis, the MMC-hypersensitive UT-SCC-12A, UT-SCC-20A, UT-SCC-24B, UT-SCC-45 and UT-SCC-60B cell lines showed a G2-block that was comparable to the FA-positive controls. The IC_{50} for MMC-induced cell killing correlated strongly with induction of a G2-block ($p < 0.0005$) (Supplementary Figure 1B). Proliferation ultimately exposes the cytotoxicity of MMC-induced DNA crosslinks through replication attempts. This could therefore affect drug sensitivity values in a manner that is unrelated to repair efficiency. However, we found no such association between the MMC-induced cytotoxicity or the G2-block and S-phase content (Supplementary Figure 2), further illustrating the value of individual cell

doubling time adaptation and choice of long term survival assay. Taken together, both tests show functional defects in cellular DNA crosslink repair in a significant proportion of HNSCC cell lines.

Hypersensitivity to the PARP inhibitor olaparib supports functional DNA repair defects

PARP inhibitors have been shown to reveal FA/HR-defects, by inducing kill in repair-defective cells [5,26]. We therefore tested our HNSCC panel for sensitivity to the PARP inhibitor olaparib (AstraZeneca). Olaparib response varied highly (Supplementary Figure 3). IC_{50} was not reached at the highest tested dose in eight of the cell lines. As reported previously, FA-cells were hypersensitive to olaparib [26] and define the lower limit in this sensitivity range. Several HNSCC cell lines were indistinguishable from these FA-patient derived fibroblasts, strongly indicating functional FA/HR-pathway defects in these lines. No correlation was found between S-phase content or doubling time and olaparib (Supplementary Figure 4A).

We did not find a strong correlation between MMC and olaparib response (Figure 1D, Supplementary Figure 4B). Consistent with the existence of olaparib resistance mechanisms unrelated to or bypassing HR-mediated repair, some MMC-hypersensitive cell lines, the UT-SCC-12A and UT-SCC-60B, did not exhibit a concomitant olaparib hypersensitivity [27]. Notably, all highly olaparib-sensitive cells were also MMC-hypersensitive, further confirming a functional FA/HR-pathway defects in these cell lines.

Aberrant FANCD2 expression and mono-ubiquitylation point to FA-pathway defects

Mono-ubiquitylation of FANCD2 is an essential step in FA-pathway activation upon DNA damage [27,28]. A lack of MMC-induced FANCD2-L demonstrates FA-pathway defects upstream of the ubiquitylation event. We tested the FANCD2-ubiquitylation capacity in 18 HNSCC cell lines and found that anomalies are common. FANCD2 expression varied and was very high in UT-SCC-43A (Figure 2, Supplementary Figure 5). Only three cell lines responded to MMC treatment with an increase in FANCD2-mono-ubiquitylation. Despite a strong MMC cell survival assay response, UT-SCC-45 and UT-SCC-43A lack efficient FANCD2 mono-ubiquitylation at any condition. These data further support DNA damage response irregularities and FA-complex defects leading to disrupted FA-pathway activation in HNSCC.

FA expression analysis reveals lack of FANCF expression in one HNSCC cell line

The observed FA phenotype-like properties of a proportion of the HNSCC prompted us to search for the genetic cause. FANCF down-regulation mediated by promoter methylation has been reported in HNSCC [18]. We therefore determined FANCF expression by PCR and tested whether it was associated with the observed functional defects. No correlation or cut-off analysis supported a role of FANCF expression in defining MMC sensitivity (Figure 3A). However, the FANCF expression level was undetectable in UT-SCC-43A and is consistent with its lack of MMC-

induced FANCD2-ubiquitylation, thereby revealing the likely cause of the observed FA-pathway defect in this cell line. RNA-sequencing analysis in all cell lines confirmed the lack of FANCF expression in UT-SCC-43A but did not reveal additional hits.

DNA sequencing identifies FA/HR gene variants with a functional association

To further uncover genetic defects that may explain the above observed crosslink repair defects, we performed capture-based sequencing including 27 canonical FA/HR-pathway genes (Supplementary Table 2). Copy number analysis on the sequencing data did not reveal homozygous deletions of the FA/HR genes. We next called single nucleotide variants and small indels and applied a variant selection protocol that was designed to enrich for functional alterations (Supplementary Table 3). In brief, considering the requirement for loss of heterozygosity (LOH) for most mutated DNA repair genes to affect cellular function, it selects for homozygous non-synonymous variants with a low or lacking minor allele frequency with the aim to enrich for variants and mutations with a potential functional association. Confirming the specificity of this approach, the known *FANCA* and *FANCG* germline mutations in the FA-patient derived fibroblasts were identified and no additional variants were selected. No FA/HR gene variants, as defined by our selection criteria, were found in the GM847 normal fibroblasts.

The analysis revealed seven FA/HR-variants in seven HNSCC lines: three in *BRCA1*, two in *FANCD2* and one in *BRCA2* and *BRIP1* (Figure 3B). With the exception of UT-SCC-30, these variants exclusively occurred in the MMC-hypersensitive cell lines, supporting their potential role in identifying crosslinker sensitivity (Figure 3C). Consistent with an FA/HR defect and the MMC and olaparib hypersensitivity endpoints, the suspected BRCA1 mutation carrier UT-SCC-60B showed impaired radiation-induced rad51 foci formation (Supplementary Figure 6). In contrast, the resistant UT-SCC-30 did not show any apparent HR pathway defects (Supplementary Figure 6), suggesting a false positive assignment by the variant selection protocol. We next used the *in silico* algorithms PolyPhen [29] and SIFT [30] to predict deleteriousness of the variants. Five of these seven variants were predicted to be damaging or deleterious (Supplementary Table 4). While among those variants some are rare or moderately rare SNPs, two of these variants, those in *BRIP1* and *FANCD2*, are unreported in the 1000 Genomes database. The location and nature of these variants strongly suggests an impact on protein function. The *BRIP1* variant Gly690Arg is located within the helicase domain and is strongly predicted to affect *BRIP1* protein function (Figure 3B, Supplementary Table 4). The *FANCD2* mutation affects a proline flanking a highly conserved region that encompasses the heterodimer interface, resulting in a deleterious prediction by PolyPhen. The other *FANCD2* missense variant is reported in the 1000 Genomes database and is located within highly conserved regions of the DNA binding domain. It was detected in the FANCD2-mono-ubiquitylation defected UT-SCC-45, indicating a causative link.

LOH is common in tumors of carriers of pathogenic BRCA1/2 germline variants that predispose to breast cancer. This prompted us to investigate LOH in the genes in which the seven variants are located. We therefore assessed the zygosity of all sequenced SNPs in these genes (Supplementary Figure 7). We found that all 19 *BRCA1* SNPs detected in UT-SCC-38 are homozygous, strongly suggesting LOH of the potentially mutated *BRCA1*. Similar evidence of full or partial LOH of *BRIP1* and *BRCA2* is present in the UT-SCC-12A and UT-SCC-76A respectively. Two out of three *BRCA1* variants in UT-SCC-60B are homozygous and have a minor allele frequency (MAF) below 5%, also pointing to potential LOH events. We were not able to assess LOH events using SNPs in the other three cell lines due to low SNP density, however, copy number data point to LOH in the respective genes in two of them, UT-SCC-15 and UT-SCC-45 Supplementary Figure 7).

Some of the selected FA/HR-variants, due to the selection criteria, may just expose LOH events, rather than being causative. Their incidence in the repair-defected cell lines, however, suggests a functional link and therefore a potential role as repair defect markers. Therefore, we evaluated whether all the variants (including possible false positive, i.e. the UT-SCC-30) were associated with MMC-sensitivity. The 'FA/HR-gene affected' HNSCC cell lines (i.e. variant-positive) were significantly more sensitive than the non-mutated (Figure 3D, $p < 0.05$). Likewise, MMC-hypersensitive were significantly enriched for FA/HR-pathway variants (Supplementary Figure 8A, $p < 0.005$). This association did not originate from a higher mutation load in these cell lines and was specific to the FA/HR-pathway further supporting the bioinformatics selection approach (manuscript in preparation). We analyzed 17 additional crosslink repair and DNA damage response genes that act in the periphery of the FA/HR-pathways and found one additional rare variant in *ATR* (Supplementary Figure 8B,C) in the MMC-sensitive UT-SCC-14, hence improving the MMC-sensitivity association ($p < 0.01$; Supplementary Figure 8D).

Taken together, we identified genetic variants in the FA/HR-pathway that are associated with functional repair defects.

FA/HR gene variants are present in a considerable proportion of HNSCC tumors

Encouraged by the strong association of the FA/HR-variants with the functional crosslink repair defect *in vitro*, we investigated the presence of such variants in a cohort of 77 advanced stage oro- and hypopharyngeal HNSCC tumors from chemo-radiated patients (Table 1). Since associated with function, we applied the variant selection protocol that was used for the cell lines, while correcting for stromal contribution in order to enrich for homozygous variants. 19% (15/77) of the HNSCC, a fraction similar to the cell line panel (24%), possessed such FA/HR-variants (herein termed 'FA/HR-affected' tumors). Figure 4A and Supplementary Table 5 summarize and list these variants, their predicted alterations and distribution over the patient cohort. Half of these variants were predicted to be deleterious or damaging by PolyPhen and

SIFT or were referenced in COSMIC. We further found a higher prevalence of rs17885240 in our patient cohort than in the general population (4/77 versus MAF = 0.0129; $p < 0.05$). Others have shown a high prevalence of rs17885240 in childhood AML (Supplementary Table 5).

FA/HR gene variants with in vitro functional association are associated with poor outcome in HNSCC

Due to the functional repair defect association of the FA/HR gene variants *in vitro*, we next questioned whether the identified patients had a different prognosis. The known and reported HNSCC prognostic variables tumor site, tumor volume and HPV-status affect outcome in our patient cohort (Supplementary Figure 9A-F) [12]. We next compared the clinical outcome of patients with 'FA/HR-affected' tumors to the others, while adjusting for these known prognostic variables in a Cox proportional hazards model. We find that the overall survival (OS) of patients with 'FA/HR-affected' tumors is lower (multivariate HR 2.6; 95% CI 1.1-6.0; $p < 0.05$) (Figure 4B, Supplementary Table 6). Locoregional control was worse in these patients (univariate HR 2.1; 95% CI 0.5-8.1; $p = 0.21$ and multivariate HR 3.5; 95% CI 0.8-15.2; $p = 0.09$) (Supplementary Figure 10), but this did not reach significance. This was likely due to the small number of locoregional events (n = 10) and also prevents meaningful conclusions from multivariate Cox model analyses that aim to account for the three major clinical variables. There was no apparent association between HPV-status and the presence of FA/HR-variants (Supplementary Table 7). Standard somatic FA/HR-variant selection did not reveal any outcome association, further highlighting the value of FA/HR-variant selection criteria and the functional *in vitro* analysis (Supplementary Figure 11).

Next, we evaluated indirect or FA/HR unrelated elements that may have driven the observed association of the FA/HR-affected tumors with poor patient outcome. Mutation load was not of influence, since these tumors have similar variant and mutation frequencies to the other tumors (Supplementary Figure 12). 529 additional HNSCC and cancer related genes were sequenced in these tumors. To confirm specificity to the FA/HR pathways, we then assessed how likely the HR of 2.6 that was found in the 'FA/HR-affected', is, by applying the FA/HR gene variant selection protocol to 10.000 random gene sets of similar total base coverage (Figure 4C). Only 304/10.000 random gene sets have a HR > 2.6, confirming the significance of the association between HR/FA-variants and the poor outcome. Finally, our variant selection approach may have marked LOH of larger chromosomal segments associated with poor outcome. We therefore repeated our analysis on other genes in the same chromosomal location and did not find a significant association with overall survival or locoregional control (Figure 4D, Supplementary Figure 10B, Supplementary Table 6 and 8) further confirming specificity to the FA/HR-pathway genes.

Not all patients received equally high cisplatin doses, either due to discontinuation or a planned low dose cisplatin chemo-radiation scheme. True (functional) FA/HR-pathway disruption would

imply an increased tumor sensitivity to crosslinking agents. We therefore further grouped patients according to their received cumulative cisplatin dose (Figure 4E). In line with the prediction, only FA/HR variants harboring tumors responded to a change in cumulative dose and our data show a benefit from high cumulative cisplatin doses for these patients while failing to show this in the non-FA/HR affected. In summary, we find that FA/HR-pathway variants and mutations mark FA/HR repair defects *in vitro*. Identifying such variants in HNSCC patients allowed us to reveal a worse prognosis group, suggesting that such tumor repair defects have an impact on patient outcome.

DISCUSSION

Prompted by the relevance of genetic HR/FA repair defects in breast cancer and the high incidence of HNSCC in FA-patients [7,8], we investigated the role of such defects in sporadic HNSCC. As similar genetic markers were missing, we first determined the incidence and properties of functional DNA repair defects by performing multiple functional assays on 29 HNSCC cell lines. We find that a significant proportion exhibit DNA crosslink repair defects comparable to FA-patient derived fibroblasts, as determined by the functional endpoints MMC-hypersensitivity, G2-blocks, olaparib-hypersensitivity and FANCD2 mono-ubiquitylation. Our comprehensive large cell line panel data therefore shows the high prevalence of functional DNA crosslink repair defects in HNSCC in vitro. We next determined possible genetic causes with the intention to translate these findings to the clinic and find multiple FA/HR gene variants that are associated with the functional outcome parameters. When retrospectively determining FA/HR gene variants with such an *in vitro* confirmed functional relevance in clinical samples, we find an incidence of 19%. Importantly, patients with tumors that harbor such genetic markers, do worse. No other genetic pathway analysis was able to depict this worse prognosis patient population, thereby further supporting the important role of such potential repair defects in these cases.

Other *in vitro* studies indicated crosslink repair defects in HNSCC. A large variation in response to the crosslinker cisplatin in ten cell lines tested by Snyder et al [31] supports our conclusion. We find at least two HNSCC cell lines that are incapable of mono-ubiquitylating FANCD2 (UT-SCC-45 and UT-SCC-43A) indicating a defect upstream or in FANCD2 itself. In both cell lines this was consistent with the genetic defect. This low incidence is consistent with other smaller studies and explains the lack of disrupted FANCD2 mono-ubiquitylation or FA-gene expression deregulation in the study of Snyder et al. Also Burkitt [32] observed defective FANCD2-foci formation in three cisplatin sensitive HNSCC. The authors found decreased BRCA1 expression and showed this caused cisplatin sensitivity in one cell line. Furthermore, MMC and cisplatin-responsive HNSCC cell lines have been found by a chromosomal breakage assay [22]. No other study has comprehensively tested for both functional and genetic FA/HR-defects in such a large

panel of HNSCC cell lines. Some studies sought after genetic defects in functional affected only, others employed endpoints, such as FANCD2-ubiquitylation, that do however not capture repair defects downstream of the event.

The discovery of frequent FA/HR defects may have also been impeded by weak IC50 values that were determined by short term metabolic assays in other studies. Our HNSCC cell line panel exhibited a large range of doubling times (17h to 79h, Supplementary Table 1) providing sensitivity and specificity issues in standard short term (72h) survival assays. Our long term and doubling time adjusted survival assay was able to uncover MMC sensitivity in slow HNSCC with DTs of over 48h. Yet, other HR/FA unrelated factors can affect some of the endpoints on an individual basis and therefore demands multiple endpoint analysis. The response to MMC partly depends on metabolic activation by cellular reductases such as the NAD(P)H:Quinone oxireductase-1 (NQO1) and the extent of glutathione detoxification [33,34]. However, these processes rarely affect the response by an enhancement factor of more than three [35–37]. It should be noted that such factors could also affect the apparent FANCD2-ubiquitylation response at a given MMC concentration. Rad51 foci induction by radiation, however, reflects the ability to engage HR [38]. Consistent with its resistance to MMC and olaparib, this response therefore excludes a BRCA defect in UTSCC-30 (Supplementary Figure 6). Apparent Olaparib resistance is possible in the presence of FA/HR defects. This can be the result of an increased expression of the drug efflux transporters *Abcb1a/b* genes, which encode the P-glycoprotein [27]. Moreover, recent studies show a role for p53-binding protein 1 (53BP1) and REV7 for olaparib resistance in BRCA1 deficient cells [39,40]. Notably and consistent with our observation, the authors report olaparib resistant tumors which maintained crosslinker cisplatin sensitivity [40]. While FA/HR-defects can therefore not be excluded in olaparib resistant HNSCC or in HNSCC with somewhat higher MMC IC50 values, the manifestation of the combined olaparib and MMC hypersensitivity is a strong indicator of functional crosslink repair defects. By our multiple endpoint analysis we were able to unmask multiple FA/HR defects. At least a quarter of HNSCC exhibited strong FA/HR defects as determined by multiple endpoints. Any cut-off for classification would be however arbitrary, clearly evident from the distribution shown in Figure 1B or Figure 3C and many more HNSCC show signs of repair defects.

Aided by the quantity of different cell lines and endpoint analyses in our study, we were also able to define genetic and repair defect associations ultimately providing a variant selection protocol that was designed to enrich for variants with a potential functional association. The selection of homozygous variants and rare SNPs are noteworthy elements of our variant selection protocol. First, we reasoned that functional DNA repair defects will generally be enabled or caused by the loss of the functional allele and therefore only selected homozygous variants. LOH of three FA genes has recently been reported in sporadic HNSCC (33). As shown here, our

approach can also capture and enrich for potentially relevant LOH events in the case of rare SNPs. Thus we acknowledge that these particular variants may not have a direct impact on the gene function. A related challenge in evaluating the consequence of individual missense variants was the absence of matched normal samples. Selection of homozygous variants does also not exclude an effect on repair of potential heterozygous compound mutations which are difficult to evaluate. Second, and different from other studies that discard SNPs, we included SNPs with a MAF < 2.5%. This allows for increased LOH event detection and is also based on our hypothesis that a fraction of HNSCC patients may bear hypomorphic FA/HR germline variants [41] that are pathogenic only when exposed to high levels of crosslinkers. HNSCC patients are often heavy drinkers and smokers and exposed to DNA crosslinking chemicals in both. From demographic data we estimated that 5-10% of all heavy alcohol and tobacco users will develop HNSCC [42,43]. In breast cancer, a single pathogenic BRCA1/2 variant can reach a penetrance of more than 50% [44]. Hence, we estimate that if HNSCC would be promoted by few SNPs, these SNPs could be present in up to 2.5-5% of the general population, thus used this value as cut-off. These estimates highlight how SNPs with such a relative high MAF could contribute to a HNSCC predisposition but remain masked as they would not harm the majority of carriers with little exposure to the carcinogens. Due to the heavy exposure to crosslinking agents we also expect that the type and nature of "pathogenic" FA/HR variants is largely different from those causing breast cancer or Fanconi Anemia. Indeed, we find FA/HR gene variants that have not been classified as pathogenic in hereditary breast cancer or Fanconi Anemia. Yet, some show indications of a potential disruptive nature (FANCD2 P834A, RAD51C G264S). Other variants may have simply marked LOH events in these cells and patients. Importantly, the identified variants by the chosen selection criteria were strongly associated with MMC-sensitivity, thus providing a functional link.

Homozygous variant detection is challenging in patient tumor samples due to unknown tumor sample purity, ploidy and intra-tumor heterogeneity [45]. Corrections for stromal contributions were made, using the pathologist's tumor purity estimate. Yet, in tumor samples with high stroma contribution of 40-50%, concessions had to be made ultimately allowing for a higher degree of false positives with regards to the homozygous status of the selected variants. However, when analyzing genetic data of 77 HNSCC tumors, and classifying patients according to tumor FA/HR-variant presence as FA/HR-affected we found that patients with such tumors had a worse prognosis. This worse outcome is analogous to reports in breast cancer studies, in which confirmed pathogenic BRCA1/2 mutations were associated with a more malignant phenotype and a worse prognosis [46,47]. Chromosomal and genetic instability promoted by the DNA repair defect and the associated tumor heterogeneity could be the driving force for the malignant phenotype and the apparent treatment failure in such patients. Since such tumors are hypersensitive to crosslinking agents *in vitro*, future studies will have to determine whether this

specific patient group do benefit from crosslinker-based treatment. HNSCC patients benefit from high cumulative cisplatin doses [48]. Our preliminary analysis on our initial cohort data shows that this benefit may very well be based on tumor DNA repair defects (Figure 4E). Although belonging to a poor prognosis group, the patients with FA/HR variants containing tumors were the only ones who appeared to benefit from cisplatin dose intensification (high dose vs low dose) thereby establishing survival rates as high as in the other patients.

The accomplishment of personalized medicine requires the discovery and identification, by functional and genomic approaches, of processes that could define treatment options. We assessed, quantified and characterized the FA/HR DNA repair pathway defects and probed their clinical relevance. FA/HR genomic variant selection that was supported by the functional *in vitro* analysis helped to reveal an association with poor survival in a cohort of chemo-radiated HNSCC patients and thereby points to the prognostic value of DNA repair defect identification in cancer. HNSCC has a dismal prognosis, particularly when diagnosed at an advanced state. Our data stress the relevance of repair defects in establishing such bad prognosis and reveal clinical treatment options at the same time since these defects are associated with the benefit of high cumulative doses of crosslinking agents.

Together, our data suggest a novel role of FA/HR-pathway aberrations in both sporadic HNSCC etiology and prognosis. To our knowledge this is also the first report that shows how a comprehensive *in vitro* functional and genetic DNA sequencing-based pathway analysis can reveal or enrich for functional and therefore relevant genetic alterations, thereby educating clinical marker selection strategies for biomarker development. The study enabled us to depict a subpopulation of patients with a bad prognosis that might be associated with genomic instability features through the depicted DNA repair defects. In the context of precision medicine, these defects can be exploited for tumor-targeted therapy options (e.g. PARP inhibitors) or personalized cancer treatment with crosslinking agents [6,49–51].

MATERIAL AND METHODS

Cell lines and cell culture

Head and neck cancer cell lines were established at the University Hospital in Turku (UT-SCC; listed in Supplementary Table 1), Finland and at the Netherlands Cancer Institute (NKI-SCC-263) [52–54]. 23 HNSCC are TP53 mutated and only UT-SCC-45 is confirmed TP53 wildtype. Cells were grown and assayed under low oxygen (4%) conditions and were cultured under standard culture condition at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids (Invitrogen).

Patients and clinical cohort

Pre-treatment tumor samples were obtained by biopsy from patients enrolled in our hospital from 2001 to 2010 and after documented informed consent. Patient and tumor characteristics are listed in Table 1. All patients were treated with concurrent cisplatin-based chemoradiotherapy (70Gy in 35 fractions). Different cisplatin regimens were administered: daily (6mg/m^2 intravenously), three-weekly (100mg/m^2 intravenously), or weekly for the first 4 weeks of radiotherapy (150mg/m^2 intra-arterial). Final cumulative cisplatin doses were recorded and patients were categorized into low (<300mg/m^2) and high (≥300mg/m^2) dose categories (Table 1). Tumor volumes were assessed on RT treatment planning CTs. Two categories (0-30 and >30cc) were established and considered in multivariate analyses. Time to locoregional recurrence was calculated from the start of treatment until the first of the following events: local or regional recurrence (event), death or last follow-up (censored). Overall survival time was calculated until death (event) or until the last follow-up (censored). HPV status was determined by capture-based sequencing and validated using p16 and p53 IHC and PCR on suspected positive cases.

G2 block analysis

Cells were cultured in 6-well plates for three days and treated with the indicated doses of MMC (Sigma-Aldrich) for 2 hours. After MMC treatment, cells were washed with PBS and incubated with fresh non-drug-containing medium. 48hrs later cells were prepared for flow-cytometry analysis (FAC Scan, Becton Dickinson, San Jose California USA) and re-suspended in PBS containing PI (propidium iodide, 10µg/ml) and RNAse (0,02mg/ml). Cell cycle phase distributions were measured on the PI histograms using the software CellQuest (Becton Dickinson).

MMC sensitivity analysis

Cultures were exposed to different concentrations of MMC (Sigma-Aldrich) after 1 day of culture. Cultures grew until the untreated cells had undergone at least five population doublings. Sub-confluent status was tested with internal linearity controls. Live cells from 3-6 sub-confluent wells were counted with a CASY cell counter (Schärfe system, Scotch plains, New Jersey). Survival is the fraction of the number of treated to untreated cells in %. IC_{50} values (MMC concentration with 50% growth inhibition) were calculated from third-order polynomial curve-fits on the growth inhibition values of the individual experiments.

PARP inhibitor sensitivity analysis

PARP inhibitor olaparib (formerly AZD2281/KU-0059436; provided by AstraZeneca) sensitivity was determined by a long term growth assay using the CyQuant-Cell-Proliferation Assay Kit (Invitrogen) that measures DNA content after cell lysis. Cells were cultured for one doubling-time before exposure to various doses of olaparib while adjusting DMSO concentration in all. Cells were then cultured for a period of at least 5 population doublings. Internal plate controls test

linearity and assured sub-confluent status within each experiment. Survival was determined as the fraction of the value in the treated samples compared to the untreated using a TECAN-infinite fluorescence plate reader. IC_{50} values were determined from polynomial curve-fits on the data.

FANCD2 mono-ubiquitylation analysis

Cells were treated for six hours with or without 100nM MMC two days after plating and harvested for lysate preparation. Proteins were extracted, resolved by SDS-PAGE using a gradient gel (4-15%; Bio-Rad Laboratories) and detected by anti-FANCD2 (1:500 (FI17) Santa Cruz Biotechnology, Santa Cruz) or anti-β-actin IgG (Sigma, 1:50.000), IRDYE 680/800 conjugated anti-mouse or anti-rabbit IgG (Licor, 1:7500). Quantitative analysis of FANCD2-L (long and mono-ubiquitylated) and FANCD2-S (short) of two to three independent experiments on multiple blots each was done on the LICOR platform (Biosciences) and expressed as L/S ratio.

FANCF expression analysis

RNA from exponentially growing cells was isolated according to standard protocols. FANCF RT-PCR analysis was performed in the 7500 Fast Real-Time PCR system (Applied Biosystems) using the primers listed in Supplementary Table 9. Samples were assayed in triplicate in at least 3 independent RT-PCR reactions and runs. FANCF expression values were normalized to two housekeeping genes. The 7500 Fast system SDS software and the 2-(ddCt) method was used for data analysis. FANCF expression in UT-SCC-43 was undetectable in all independent samples despite high RNA quality values (RIN10) and average housekeeping gene expression. The lower limit for detection was assigned to this line for calculation purposes.

HNSCC material and DNA isolation

Cellular DNA of the HNSCC cell lines and from fresh frozen tumor material was isolated using the Qiagen AllPrep DNA/RNA Mini Kit. Only material with an average tumor content of 50% and higher, as determined by a pathologist on H&E sections adjacent to and in the midst of the sections collected for DNA sequencing, was included. Researchers and bioinformaticians were blinded to patient information and outcome data. Clinicians were blinded to sequencing data. Clinical variables data and anonymous outcome data were applied after sequencing and variant selection.

DNA capture and sequencing

Paired-end (PE) fragment libraries were prepared using a genomic DNA library preparation kit (Illumina). The libraries were hybridized to a SureSelect custom-based bait library (Agilent) designed to capture exonic regions (with a 50bp extension on both sides). After washing, the captured DNA was amplified. Enriched libraries were barcoded, pooled and sequenced on a GAII (Illumina Hiseq-2000) using a 2x75bp PE protocol. Sequencing reads were aligned to

the GRCh37.55 Ensembl human reference genome using the Burrows-Wheeler Aligner 0.5.10 backtrack algorithm. Potential PCR duplicates were removed using picard-tools MarkDuplicates. An average read depth of 247 in the cell line samples and 255 in the tumor samples was achieved. Copy numbers were inferred from the DNA-seq data using PropSeg on the cell line panel and CNVkit in the tumor cohort [55,56].

Variant and mutation calling

Variants, single nucleotide variants (SNVs) and indels, were called with VarScan 2.3.9 using Samtools mpileup 0.1.19 [57]. Next, we annotated these variants with the RefSeq and 1000 Genomes august 2015 databases using Annovar version date 11-05-2016 [58]. CADD [59], PolyPhen [29], REVEL [60] and SIFT [30] were used for *in silico* variant effect predictions. Supplementary Table 3 describes the filtering steps of the variant selection protocol that was designed to enrich for variants with a functional impact. In brief, variant selection considered variant allele frequency (VAF > 0.8) and rare SNPs (MAF < 2.5%). While maintaining VAF criteria for selection, VAF values in the tumor samples were adjusted to correct for the stromal contribution. Taking into account the pathologist's tumor fraction assessment (TF), the VAF values from the sequencing data were corrected as follows to compute a VAF value for the tumor (VAF_T):

$$VAF_T = \frac{VAF - VAF_S \times (1 - TF)}{TF}$$

Matched normal tissue blood samples were largely unavailable. Stromal VAF (VAF_S) was therefore set to 0.2, thereby providing a minimal VAF value for heterozygosity.

Statistics

All analyses were performed in the R environment for statistical computing. The MMC IC_{50} values of FA/HR-variant positive versus negative cell lines were compared using a Mann-Whitney U test. A two-by-two Table holding the FA/HR-variant status of the third most MMC sensitive cell lines (n = 10) versus the other cell lines (n = 19) was analyzed with Fisher's exact test. Overall survival, locoregional control and Kaplan-Meier estimators were calculated. Multivariate Cox proportional hazard models included the tested gene set of interest and the clinical covariates tumor site, HPV-status and tumor volume. These clinical covariates have prognostic significance in HNSCC and reached significance in univariate analyses in our cohort. Further statistical analyses and values are accordingly specified in the supplementary data.

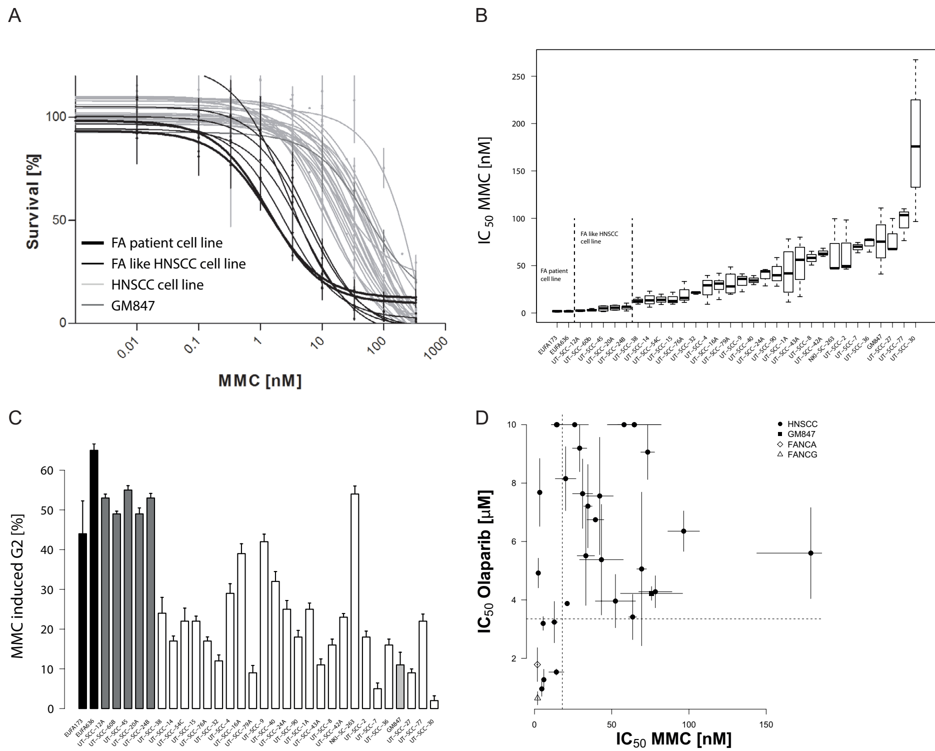

Figure 1. Sensitivity of HNSCC cell lines to mitomycin C and PARP inhibition.

(A) MMC sensitivity as measured by a prolonged growth assay. The average surviving fraction derived from three to five independent experiments per cell line. Errors are SEM. Note, MMC concentrations are log-transformed. A non-linear fit on the log-transformed data is shown. **(B)** Boxplot with MMC IC50 values in the cell line panel. Values are calculated from the curve fits on the individual experiment data and are the average of three to five independent experiments. **(C)** G2/M cell cycle phase arrest 48hrs after 1µM MMC treatment. Cell lines are ranked according to their MMC IC50. MMC-induced G2 values are corrected for the untreated. Errors are SEM. **(D)** Comparison of MMC and olaparib sensitivity in the HNSCC cell line panel. The graph demonstrates the lack of MMC-resistant but olaparib hypersensitive cell lines. Olaparib IC50 values were determined on the individual curve-fits of three to five independent experiments. Errors are SEM.
“FA-like” have been highlighted for presentation and cross-comparison purposes and depicts HNSCC cell lines with MMC IC50 values that are not significantly different from those of the FA-patient cell lines (EUFA173 and EUFA 636) that served as positive controls.

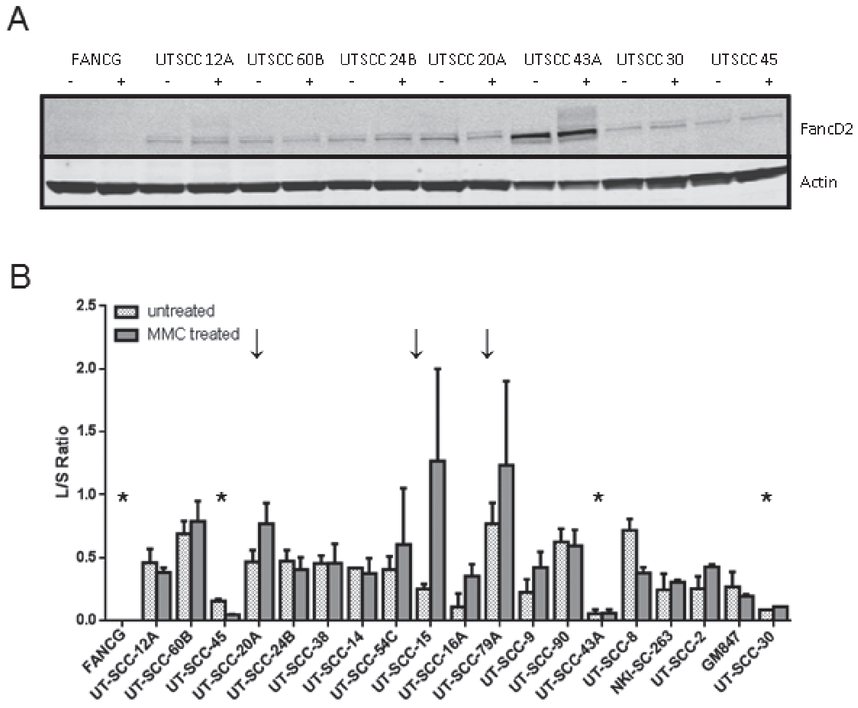

Figure 2. FANCD2-mono-ubiquitylation capacity in the HNSCC panel.

FANCD2-mono-ubiquitylation ability was assessed by exposure to MMC**. (A)** Representative example of FANCD2-ubiquitylation western blot analyses are shown. Lysates were prepared from untreated (-) or MMC-treated cells (+) 6h after treatment. The lack of the upper band indicates a lack of the mono-ubiquitylated form of FANCD2 (FANC2-L) and a defect upstream in the Fanconi pathway. Actin served as a loading control. **(B)** Quantification of MMC-induced FANCD2-mono-ubiquitylation in the HNSCC panel. Quantified FANCD2-L/S values in untreated (dotted bars) and MMC-treated (solid bars) samples of each analyzed HNSCC cell line are shown. HNSCC values are ranked according to their MMC sensitivity. Errors are SEM. Stars (*) indicate examples with an overall lack of FANCD2-ubiquitylation, arrows (↓) in contrast depict HNSCC with a pronounced MMC-induced FANCD2 mono-ubiquitylation as expected by a fully functional pathway.

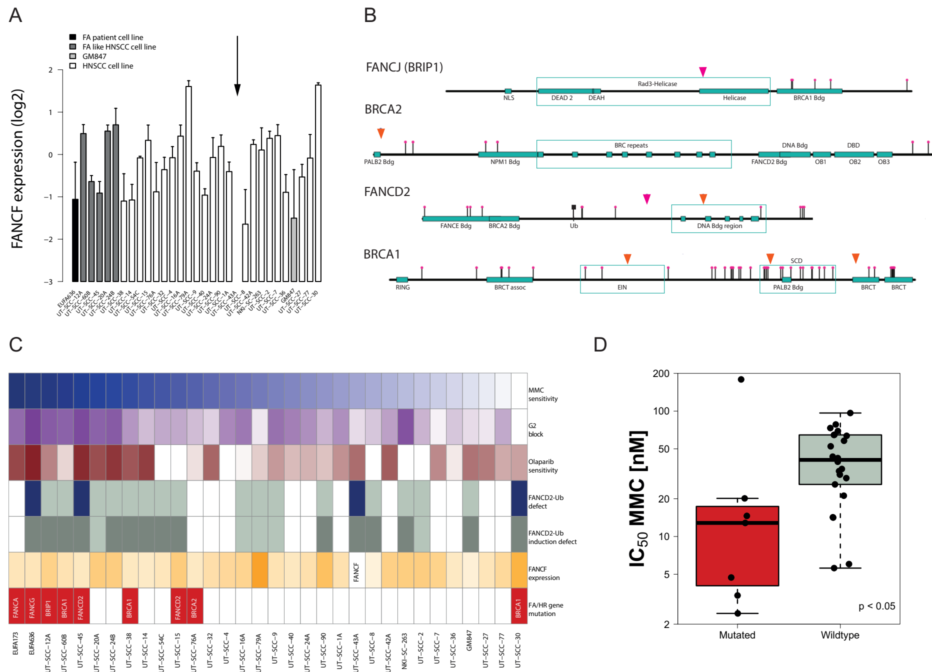

Figure 3. Identification of genetic FA and HR pathway alterations and their association with a functional repair defect.

(A) FANCF expression in the HNSCC cell line panel. HNSCC cell lines are ordered according to their MMC sensitivity. The relative FANCF expression in the individual HNSCC cell lines is shown as a deviation from average (log2-transformed) after normalization to the two housekeeping genes. Arrow (↓) depicts lack of FANCF expression. Errors are SD on the means of 3 to 5 independent PCR reactions. **(B)** Identification of potential FA and HR gene mutations in HNSCC. Homozygous rare sequence variants were found in BRCA1, two in FANCD2 and one in BRIP1 (FANCJ) and BRCA2 in 7 of the 29 HNSCC. Rare SNPs are depicted in orange, unreported non-synonymous variants in pink. **(C)** Comprehensive summary of the HNSCC DNA repair defect data. HNSCC cell lines are ordered according to their ranking in MMC sensitivity (top panel). MMC sensitivity, MMC-induced G2 block and Olaparib sensitivity are represented by a color grading with darker colors representing defects in those parameters. Blue bars display defects in FANCD2 mono-ubiquitylation and grey bars represent a lack of induction by MMC (white bars = not determined). FANCF bars are color-ranked according to their expression values. Red bars demonstrate the identification of DNA sequence variants (as shown in B). **(D)** MMC sensitivity of HNSCC with FA/HR gene variants (in red) compared to HNSCC in which such variants could not be found ($p < 0.05$).

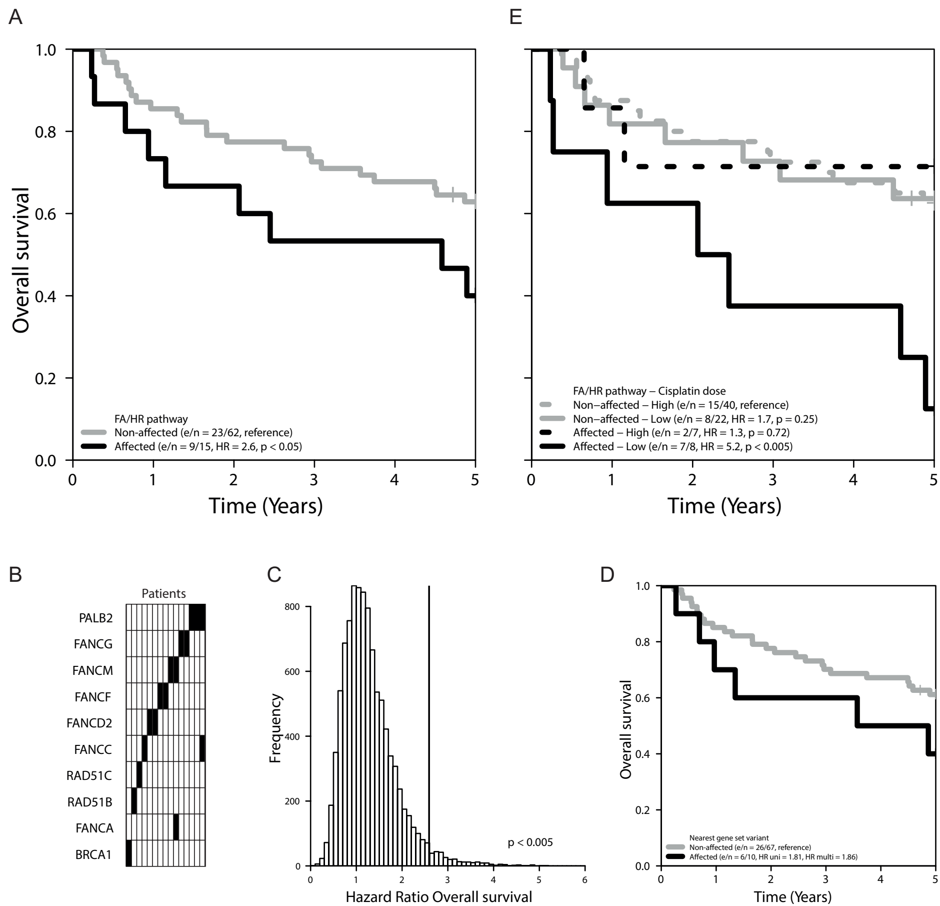

Figure 4. FA/HR gene variants in tumors of HNSCC patients and their prognostic value.

(A) Nineteen FA/HR gene variants were found in fifteen patient samples (columns), resulting in a 19.5% incidence rate (Suppl. Table 5). **(B)** Oro- and hypopharyngeal tumor samples of seventy-seven chemoradiated HNSCC patients were analysed for variants in canonical FA/HR genes by applying the variant selection criteria that returned a functional DNA repair defect association *in vitro*. HNSCC patients with FA/HR gene variants in the tumors (= FA/HR-affected) had a worse overall survival (OS) ($p < 0.05$ in multivariate analysis), demonstrating the impact of potential functional FA/HR repair defects in this patient population. **(C)** Distribution of OS hazard ratios, obtained by repeating the analysis of Figure 4A on ten-thousand randomly selected genes sets of similar base coverage, highlights the significance and specificity of the FA/HR gene variants HR finding. Gene sets were sampled from 529 sequenced cancer-related genes. **(D)** Kaplan-Meier graph showing OS of patients with variants in genes in FA/HR gene chromosomal locations. No significant association was found. **(E)** OS is worst in patients with functionally associating FA/HR gene variants and low cumulative cisplatin dose (HR 5.2, $p < 0.005$). In-figure: multivariate hazard ratios (HR) with confidence intervals (95% CI) from multivariate Cox proportional hazard models that include the tested gene set, tumor site, HPV-status and tumor volume.

Table 1. Demographics of HNSCC patient cohort.

Pretreatment biopsy material from HNSCC tumors of 77 patients was sequenced and tested for functional repair associated FA/HR-variants, as determined in the cell line panel. All patients received concurrent cisplatin-based chemoradiotherapy, with some patients reaching a high cumulative cisplatin dose of ≥ 300 mg/m2.

Patient characteristics		**N (%)**
Gender	M	55 (71)
	F	22 (29)
Primary site	oropharynx	49 (64)
	hypopharynx	28 (36)
T-stage	T1	1 (1)
	T2	13 (17)
	T3	35 (46)
	T4	28 (36)
N-stage	N0	10 (13)
	N1	7 (9)
	N2	52 (68)
	N3	8 (10)
T-volumes	0-30 cc	38 (49)
	>30	39 (51)
Events	Death	32 (42)
	Locoregional Recurrence	10 (13)
HPV	positive	21 (27)
	negative	56 (73)
Smoker	current	46 (60)
	former	19 (25)
	never	4 (5)
	unknown	8 (10)
Alcohol consumption	yes	47 (61)
	former-alcoholic	13 (17)
	never	8 (10)
	unknown	9 (12)
Cisplatin regimen	daily (6mg/m^2, 5 weeks)	17 (23)

Table 1: Continued

Patient characteristics		
	3-weekly (100mg/m², 3x)	46 (59)
	weekly (150 mg/m², 4x)	14 (18)
Cumulative cisplatin dose	low (< 300 mg/m²)	30 (39)
	high (≥ 300 mg/m²)	47 (61)
		time
Median age	at diagnosis	58 years (SD=9,6)
Median survival	Overall survival	63 months (SD=39)
	Locoregional control	63 months (SD=41)

ABBREVIATIONS

BRCA1/2: Breast Cancer Type 1 Susceptibility Protein 1 or 2; CT: computed tomography scans; FA: Fanconi Anemia; H&E: hematoxylin and eosin; HNSCC: Head and Neck squamous cell carcinoma; HPV: Human papillomavirus; HR: hazard ratio; HR: Homologous recombination; IC50: half maximal inhibitory concentration; IHC: Immunohistochemistry; MAF: Minor allele frequency; MMC: Mitomycin C; OS: Overall Survival; PARP: Poly(ADP-Ribose) Polymerase; PE: paired-end; RT: radiotherapy; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; SNV: single nucleotide variant; TF: tumor fraction; UT-SCC: University Turku - Squamous Cell Carcinoma; VAF: variant allele frequency;

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SUPPLEMENTARY INFORMATION

The supplementary information referenced in this chapter is freely available online at Oncotarget (https://doi.org/10.18632/oncotarget.24797 - Supplementary Files).

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CHAPTER 3

Role of variant allele fraction and rare SNP filtering to improve cellular DNA repair endpoint association

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ABSTRACT

Background

Large cancer genome studies continue to reveal new players in treatment response and tumorigenesis. The discrimination of functional alterations from the abundance of passenger genetic alterations still poses challenges and determines DNA sequence variant selection procedures. Here we evaluate variant selection strategies that select homozygous variants and rare SNPs and assess its value in detecting tumor cells with DNA repair defects.

Methods

To this end we employed a panel of 29 patient-derived head and neck squamous cell carcinoma (HNSCC) cell lines, of which a subset harbors DNA repair defects. Mitomycin C (MMC) sensitivity was used as functional endpoint of DNA crosslink repair deficiency. 556 genes including the Fanconi anemia (FA) and homologous recombination (HR) genes, whose products strongly determine MMC response, were capture-sequenced.

Results

We show a strong association between MMC sensitivity, thus loss of DNA repair function, and the presence of homozygous and rare SNPs in the relevant FA/HR genes. Excluding such selection criteria impedes the discrimination of crosslink repair status by mutation analysis. Applied to all KEGG pathways, we find that the association with MMC sensitivity is strongest in the KEGG FA pathway, therefore also demonstrating the value of such selection strategies for exploratory analyses. Variant analyses in 56 clinical samples demonstrate that homozygous variants occur more frequently in tumor suppressor genes than oncogenes further supporting the role of a homozygosity criterion to improve gene function association or tumor suppressor gene identification studies.

Conclusion

Together our data show that the detection of relevant genes or of repair pathway defected tumor cells can be improved by the consideration of allele zygosity and SNP allele frequencies

INTRODUCTION

Recent large-scale sequencing efforts stimulated oncology research and revealed a multitude of novel genomic alterations and somatic mutations in various tumor types [1–3]. These genetic studies are driven by the need to understand the processes related to tumor development and treatment response with the ultimate goal to find therapeutic biomarkers and targets for novel therapeutic approaches [4,5]. A major challenge in the clinical translation of these results is the discrimination of genetic alterations with a functional impact from the vast number of detected alterations.

While high gene mutation frequencies in tumors can point to potential oncogenes, defining a role for tumor suppressor genes (TSG) can be challenging. This is because TSG variants with a functional impact likely affect pathway performance only when the wild-type allele is lost. Variants in DNA repair genes, archetypal TSGs, are particularly difficult to evaluate. Many genes are involved in the repair of DNA damage and determine cellular survival following DNA damage. Mutations in any of these genes could influence cellular outcome following DNA damage. The multitude of genes and variants therefore hampers gene mutation detection as it is difficult to identify among them the affected gene or the pathway disrupting variant. Attempts to deduct DNA repair defects from genetic data for example for treatment response analyses suffer from the ignorance of the functional impact of many of the gene variants. Experimental validation of the functional impact of the individual variants is however time-consuming and costly. Rarity may also discourage thorough characterizations of the individual variant. An important role of genetic DNA repair defects in tumors could therefore been masked by a multitude of different, rare but functionally important, variants across many genes [6]. Computational tools that help to prioritize the potential functional relevance of genetic alterations are therefore of great importance. Many different tools, comprehensively reviewed in Eilbeck et al. and compared by Mahmood et al. [7,8], have been developed. They use different tactics and are valuable tools to assess a variant's probability to affect gene function. Algorithms that predict the effect of non-synonymous variants often consider the degree of conservation of homologous sequences and how disruptive an amino acid change is based on its physical properties [9,10]. However, these approaches may not suffice and in the presence of wild-type alleles such annotations are inapt to predict cellular pathway performance such when applied to tumor suppressor or DNA repair genes.

Here we propose a simple, complementary strategy that combines the zygosity status and allele frequency of variants in order to enrich for variants that label genes or tumor cells with a functional DNA repair defect. Germline *BRCA1/2* variants that predispose to breast cancer illustrate the potential value of such approaches. Pathogenic *BRCA1/2* variants manifest their

deleterious potential after loss of the wild-type allele, a process known as loss of heterozygosity (LOH) [11]. Homozygosity can thus be a selection criterion to enrich for variants with a functional impact, in particular those causing a loss of function or that mark LOH events. Pathogenic *BRCA1/2* variants occur at very low allele frequencies and are therefore often annotated in dbSNP. Variant selection protocols that remove dbSNP annotated variants in order to identify somatic mutations, also remove rare pathogenic variants [12]. To prevent this some studies select only variants that are very likely to impair gene function, such as nonsense and frameshift mutations. Other selection criteria question whether variants have a confirmed association with familial breast cancer. Such associations do, however, not reflect cellular DNA repair performance well; an endpoint relevant to treatment response association studies, in particular to targeted drugs such as to PARP inhibitors [13–15]. This is partly due to the rarity of these pathogenic variants. Also, hypomorphic gene mutations among rare single-nucleotide polymorphisms (SNPs) can influence DNA repair and response to a degree that affects drug response but not cancer incidence rates. These hypomorphic gene mutations as well as low penetrance variants will be removed by variant selection protocols that solely retain confirmed pathogenic variants. The retention of variants with low population frequency (rare SNPs) could address these issues and improve repair outcome association.

Here we set out to test the benefit of allele zygosity and SNP allele frequencies filters. Selection of homozygous variants and rare SNPs as selection criteria have been considered previously. These studies were either restricted to retrieving known cancer-associated variants [12] or performed exploratory analyses in multidrug screens without verifying causality [16]. In an effort to validate these variant selection criteria, we apply them in a setup that provides a functional link to the phenotypic effect of the selected, i.e. variant-marked, tumor cell lines. We focus on genes in the Fanconi anemia (FA) and homologous recombination (HR) pathways that are required to repair DNA crosslinks. Sensitivity to the DNA crosslinker Mitomycin C (MMC) drug is a hallmark of FA pathway defects and resulted in the identification of multiple FA pathway genes [17–20]. DNA interstrand crosslinks (ICL) pose critical obstacles in replication and this activates the FA core complex that is composed by ten FA pathway proteins. Together they function as a ubiquitin E3 ligase to mono-ubiquitylate FANCD2-I. This activation recruits endonucleases to cleave the DNA and allow translesion synthesis at the ICL affected DNA, ultimately however requiring members of the homologous recombination repair pathway to finalize ICL repair. Together, the FA and HR pathway has an important role in resolving mitomycinC induced ICLs. We therefore performed capture sequencing and variant calling on FA/HR genes in a panel of 29 patient derived head and neck squamous cell carcinoma (HNSCC) cell lines, of which a proportion was previously shown to have FA/HR pathway defects [21]. The defects were revealed by MMC response data and confirmed by additional crosslink repair function parameters [21]. The MMC response data provide a robust functional readout for crosslink repair that allowed us to assess the value of

combined allele zygosity and SNP allele frequency filters to detect cellular repair defects and/or relevant genes.

METHODS

Cell line panel

The following HNSCC patient derived cell lines were generated at the Turku University Hospital Finland between 1990-2002: UT-SCC-1A, UT-SCC-2, UT-SCC-4, UT-SCC-7, UT-SCC-8, UT-SCC-9, UT-SCC-12A, UT-SCC-14, UT-SCC-15, UTSCC-16A, UT-SCC-20A, UT-SCC-24A, UT-SCC-24B, UT-SCC-27, UT-SCC-30, UT-SCC-32, UT-SCC-36, UT-SCC-38, UT-SCC-40, UT-SCC-42A, UT-SCC-43A, UT-SCC-45, UTSCC-54C, UT-SCC-60B, UT-SCC-76A, UT-SCC-77, UT-SCC-79A and UT-SCC-90. The conditions under which these cell lines were cultured have been described previously [22,23]. The HNSCC cell line NKI-SCC-263 was established at the Netherlands Cancer Institute. Two FA patient derived fibroblast cell lines were provided by Dr. H. Joenje (Vrije University Amsterdam). Our sequence analyses confirmed the reported FANCG 1649delC and FANCA Arg951Gln mutations in these FA patient fibroblast line (EUFA636 [24] and EUFA173 [25]). These FA pathway mutated cell lines served as positive controls and provided the reference values in the MMC response data. These cell lines were not considered in the variant selection criteria assessment studies. Notably, all cell lines were cultured and tested under low oxygen (5%) conditions since high oxygen conditions affect cellular growth and fitness of repair defected cell lines. The negative control cell line was an hTERT transformed human fibroblast cell line (GM847), provided by Roderick Beijersbergen (the Netherlands Cancer Institute). Mitomycin C (MMC; Sigma Aldrich) sensitivities have been determined as described previously [21]. In brief, cell doublings were assessed and cellular survival after treatment with different concentrations of MMC was determined by live cell counting after multiple divisions (minimum 5) in a long-term growth assay. Controls were included with lower cell densities that tested and assured linearity in these assays. Survival was determined relative to untreated cells and the MMC concentration resulting in 50% survival (MMC IC_{50} value) was calculated for each cell line from third order polynomial curve fits on the growth inhibition curves of the individual independent experiments [14,21]. Data are from three to five independent experiments per cell line with 3 to 6 replicates each. Mean MMC IC_{50} values were used in analyses. MMC concentrations were adapted in the individual cell lines to assure a good coverage of data points in particular in the IC50 to IC90 inducing MMC dose range

Patient samples

Tumor samples were obtained after documented informed consent. Consent forms were approved by the medical ethical committee of the Netherlands Cancer Institute. Use of the material for this genetic study was approved by the institutional ART-CFMP biobank review board. Tumor samples are from 56 patients with advanced head and neck squamous cell carcinoma

(HNSCC) that were enrolled in our hospital between 2001 and 2010 and obtained from fresh frozen pretreatment biopsies. Matched blood samples were not available for these samples.

DNA capture and sequencing

Genomic DNA was isolated using the AllPrep DNA/RNA Mini Kit (Qiagen). Paired-end fragment libraries were prepared with the TruSeq DNA library preparation kit (Illumina) and target-captured with a SureSelect custom-based bait library (Agilent) targeting 556 genes (Supplementary Table A). The capture covers 1.9 Mb with about 24000 probes. Baits covered all exons (average coverage 1.5) and UTR and TSS were additionally covered in the canonical FA/HR pathway genes. DNA was washed, amplified, barcoded, pooled and sequenced on the Illumina Hiseq 2000 using a 2x75bp paired-end protocol. Sequencing reads were aligned to the human reference genome (GRCh37.55/hg19) with the backtrack algorithm of the Burrows-Wheeler Aligner 0.5.10 [26]. Potential polymerase chain reaction duplicates were removed with Picard Tools (http://picard.sourceforge.net). The average read coverage was 247. Blood samples from four healthy volunteers were sequenced to depict potential capture errors and bias and to monitor sequencing noise.

Variant calling

Variants, both single nucleotide variants and small insertions and deletions, were called with VarScan 2.3.9 [27] in conjunction with Samtools mpileup 0.1.19 [28]. VarScan's VarFreq (divided by 100) provided the variant allele fraction (VAF) values used in this study and represent the fraction of reads that show the individual variants. Single nucleotide variants were called when allele coverage was at least ten, the number of variant reads at least four, and the VAF at least 0.10. The same parameters were used for calling small insertions and deletions, except the minimum number of variant reads was set to ten with no VAF restriction. Single nucleotide variants adjacent to small insertions and deletions were removed. We used Annovar (version date 11-05-2016) [29] to annotate variants with the RefSeq and 1000 Genomes European august 2015 (Phase 3) databases [30]. SNPs refer to variants with an associated minor allele frequency (MAF) in the 1000 Genomes European database. All analyses and figures were restricted to non-synonymous exonic variants and essential splice site variants throughout this paper. Variants that occurred in all four healthy volunteer blood samples were removed as they were considered potential artifacts.

Statistical analysis

In order to assess the value of the selection criteria to detect cellular crosslink repair defects, we compared 'pathway mutated' with 'non-mutated' cell lines in various pathways and gene sets. 'Pathway mutated' cell lines had one or more variants (potential mutations) in any of the pathway genes, the 'non-mutated' had none (Supplementary Figure A). The association

between pathway mutation status and MMC response was evaluated with the Wilcoxon rank-sum test and visualized by the Positive Predictive Value (PPV). The Wilcoxon rank-sum test compares the (ranked) MMC IC_{50} values of ‘pathway mutated’ and ‘non-mutated’ cell lines. The PPV was calculated as the fraction of ‘FA/HR pathway mutated’ cell lines present among the ten most MMC sensitive cell lines (Supplementary Figure A). The number of LOH events across TSGs and oncogenes was compared with the Wilcoxon rank-sum test. Fisher’s exact test was used to compare the proportion of heterozygous and homozygous variants between TSGs and oncogenes. All statistical analyses were performed in the R environment for statistical computing.

RESULTS

Variant allele fraction filtering improves functional outcome association

Here we set out to investigate the association between the zygosity status or allele frequency of a variant and its ability to mark a phenotypic effect. To this end we employed a panel of 29 patient-derived HNSCC cell lines. We employed response to the DNA crosslinker MMC as a hallmark and readout of cellular DNA repair proficiency (Supplementary Figure B). Tumor cell DNA repair capacity is the essential functional endpoint in cancer treatment response or DNA repair targeting strategies studies. Cellular MMC response is strongly influenced by the activity of the FA and HR gene products [17–20]. Assay performance and MMC sensitivity to repair relation was confirmed by the revealed MMC hypersensitivity of FA patient fibroblast controls with confirmed FA gene mutations and lack thereof in wild-type fibroblasts (Supplementary Figure B). Capture sequencing data on 556 genes, which included the canonical FA/HR pathway genes (Supplementary Table A), was available and we performed variant calling with different parameters on those genes.

We first factored out the allele frequency by removing all SNPs present in the 1000 Genomes database and focused on the role of the zygosity status. To this end we employed variant allele fraction (VAF) as a measure of allele zygosity, with high VAF corresponding to homozygosity. The VAF distribution of all non-synonymous variants reflected allele zygosity well in these cell lines (Supplementary Figure C). We varied VAF thresholds from low to high and at each threshold value removed variants with a VAF below the threshold. Thus, each VAF threshold resulted in a unique set of variants being retained. A cell line was assigned as ‘FA/HR pathway mutated’ if any FA/HR gene variant in that cell line was retained under the tested threshold. Increasing VAF thresholds gradually reduced the number of retained variants and number of cell lines with a ‘FA/HR pathway mutated’ assignment (Supplementary Figure D). We then tested whether the MMC IC_{50} values showed an association with the ‘FA/HR pathway mutated’ status. Specifically, we performed the Wilcoxon rank-sum test and calculated the Positive Predictive

Value (PPV), i.e. the fraction of ‘FA/HR pathway mutated’ cell lines present among the ten most MMC sensitive cell lines. As control we repeated our analysis on 10.000 random gene sets, each having cumulative sequence lengths similar to the FA/HR gene set (‘Similar sized gene sets’). The association between ‘FA/HR pathway mutated’ and MMC sensitivity increased and reached significance with higher VAF thresholds (Figure 1A and Supplementary Figure D). In contrast, the PPV of the control sets remained constant at 0.3, indicating a random assignment hence a lack of discrimination power (Figure 1A and Supplementary Figure E). This indicates that the specificity of FA/HR variants to detect MMC hypersensitive, and therefore repair-defected, cell lines improves with increasing homozygosity.

Next, we assessed indirect factors that could have caused or confounded the here observed association between homozygosity of FA/HR variants and MMC sensitivity. The apparent association with homozygosity of the FA/HR variants could be an indirect consequence of an increased total or homozygous variant load in these MMC sensitive cell lines. However, neither the total nor the homozygous variant load was associated with MMC sensitivity (Supplementary Figures F and G), regardless whether SNPs were included or excluded. Homozygous FA/HR variants could act as ‘tags’ for other homozygous variants in genes in the proximity of the FA/HR genes (Supplementary Table A). However, we find that variants in neighboring genes were not associated with MMC sensitivity (Figure 1A and Supplementary Figure E, “Nearest genes”). LOH events, across a larger region neighboring and containing the FA/HR genes, are thus unlikely to have caused the association between the homozygosity of FA/HR variants and MMC sensitivity. These results show that the improved MMC response association by these filters is FA/HR gene-specific. Together, our results support homozygosity as a variant selection criterion.

Rare SNP filtering improves functional outcome association

We next investigated the benefit of considering the allele frequency of FA/HR variants for DNA repair defect marking. We factored out the variable zygosity status by only using homozygous variants. This analysis was similar to the previous, except that variants were now filtered according to their allele frequency in the 1000 Genomes database instead of their VAF in the cell line sample. We did so by varying the allele frequency, also known as the MAF, threshold (Supplementary Figure H). The number of variants and cell lines with a ‘FA/HR pathway mutated’ assignment decreased pronouncedly at the MAF threshold value of 0.1. Notably, the association between FA/HR variant marked cell lines and MMC sensitivity increased and reached significance with lower MAF thresholds (Figure 1B, Supplementary Figures H and I). This effect was neither seen in the ‘nearest genes’ nor in the 10.000 similar sized gene sets.

We further studied the interaction between zygosity and allele frequency by filtering on both the VAF and the MAF. We then evaluated the association of the cell lines classified as repair defected

by the remaining variants with MMC sensitivity. This analysis shows that the PPV increases proportionally to the VAF threshold, though only after common SNPs have been removed, i.e. at lower MAF thresholds (Figs 1C and 1D). Multiple of the VAF and MAF threshold combinations show a significant association with MMC sensitivity, further illustrating the value of a combined approach (Supplementary Figures J and K). In addition, when assessed in the two FA patient derived fibroblast cell lines, these filters identified the reported FANCG 1649delC and FANCA Arg951Gln mutations. In summary, these results show that the variant selection criteria allele zygosity and allele frequency can be combined to enrich for variants that mark cell lines with a loss of cellular repair function. Selecting homozygous variants and rare SNPs detects variants with a link to loss of pathway function in our dataset, and thus also helps to prioritize variants for further experimental validation.

VAF and rare SNP filtering improve pathway identification

The previous analyses focused on the FA/HR pathway, because defects in FA/HR genes are well-known to impair the repair of crosslinks induced by MMC. In practice, the aim of genetic analysis is often to reveal novel associations with drug response, e.g. in pharmacogenomic interaction studies [5,31,32]. Given the importance of the FA/HR pathway for the removal of MMC induced crosslinks, the prioritization of this pathway over others by variant selection strategies that consider homozygosity and rare SNPs would confirm their value.

To this end we broadened the analysis to include all genes annotated in all Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Based on the results of the previous section we selected homozygous variants (VAF ≥ 0.8) and included rare SNPs (MAF ≤ 1%, a threshold commonly used to define rare variants). We scored a pathway as 'mutated' when any of the genes annotated to that pathway were 'mutated', i.e. has a variant as selected by the above criteria. For each pathway, the MMC IC_{50} values of 'pathway-mutated' cell lines were compared to those of 'non-mutated' cell lines. Some mutational profiles of pathways correlated strongly (Pearson correlation r ≥ 0.75) because they were only represented by a few overlapping genes in our capture-set. In these cases the pathway with the weakest MMC response association was removed. The results of this analysis are represented in Figure 2A. We find that the Fanconi anemia (FA) pathway is the only pathway that is significantly associated with MMC sensitivity.

We then compared this analysis to an approach that includes all non-synonymous variants regardless of zygosity status, i.e. performs no VAF filtering. At the same time, variants and SNPs with a MAF ≤ 1% were retained. This approach was not able to reveal an association between the Fanconi anemia pathway and MMC sensitivity. This result is consistent with the requirement of loss of the wild-type, the functional DNA repair pathway gene allele, to impact cellular repair. It thereby confirms the benefit of using zygosity as a variant selection criterion to depict repair

defected cells. The analysis did, however, reveal significant associations between four other pathways and MMC resistance (Supplementary Figure L). Upon further examination we found that two of these associations were mainly driven by falsely assigned *TP53* mutation status in the cell lines. Two *TP53* mutations were missed in MMC sensitive cell lines; one point mutation remained undetected because of low read depth and one known medium sized deletion was not picked up due to limitations of short-read sequencing data (Supplementary Table B) [22,33–37]. When correcting the *TP53* mutation status these two associations disappear (Figure 2B). The other two pathways associated with MMC resistance were the Hippo signaling and mineral absorption pathways, while the Rap1 signaling pathway was associated with MMC sensitivity (Figure 2B).

We next compared homozygous and rare SNP variant selection strategies to standardly used variant selection methods such as REVEL and CADD. Variants in FA/HR genes that were selected by these methods did not show an association with the functional endpoint (MMC response) at any threshold (Supplementary Figure M). These variant selection methods did also not reveal a FA pathway link in the broader pathway analysis (Supplementary Figure N). Our proposed homozygous and rare SNP variant selection strategies however did prioritize the FA pathway over all other KEGG pathways (Figure 2A), since it had the strongest association with MMC response. As the FA pathway has an established role in cellular MMC response, this supports the value of our variant selection strategy for exploratory analyses.

Tumor suppressor genes are enriched for LOH and homozygous variants

After focusing on DNA repair genes, we next investigated TSGs in a broader sense and assessed the potential value of the homozygosity criterion for tumor suppressor gene identification in patient tumor samples. Sample tumor purity, intra-tumor heterogeneity and (aneu)ploidy hamper the ability to distinguish between heterozygosity and homozygosity based on the VAF values in tumor samples [38]. We therefore questioned whether selecting homozygous variants in tumor samples would be able to enrich for tumor suppressor genes. Homozygosity, often through the loss of a functional wild-type allele, is one of the means to accomplish the loss of the cellular function of a gene, a requirement common to TSGs. Conversely, the activating mutations in oncogenes do not necessarily require homozygosity to have a functional impact. We therefore tested whether known TSGs were enriched for LOH events/homozygous variants in patient tumor samples.

To this end, we applied the same variant calling pipeline that was used for the HNSCC cell line panel to capture sequencing data from 56 HNSCC tumor samples. Rare non-synonymous SNPs were included (MAF ≤ 1%). We find that the VAF distribution has three peaks in the tumor samples, in contrast to the bimodal VAF distribution of the HNSCC cell line panel (Supplementary

Figure O). It illustrates how the contribution of tumor purity, intra-tumor heterogeneity and ploidy to the VAF values complicates the distinction between heterozygous and homozygous variants based on VAF values. We therefore used the algorithm PureCN [39] to identify genes and variants that underwent LOH. PureCN employs VAF and read coverage to produce an improved estimate of the allele specific copy number and thus LOH. Six documented HNSCC TSGs and ten oncogenes (Supplementary Table C) were used to narrow the analysis [40]. First, we determined the number of samples with LOH per gene (Figure 3A). LOH occurred five times more often in TSGs than oncogenes (average of 27 LOH events across TSGs versus 5 across oncogenes; $p < 0.005$; Figure 3B). Second, we pooled the variants of all samples in the TSGs and oncogenes and calculated the fraction of gene variants that was homozygous. The fraction of homozygous variants was six times higher in the TSGs than in the oncogenes (37 / 68 versus 1 / 11; $p < 0.005$).

The results show that in the HNSCC tumor samples, LOH and homozygous variants are more common in TSGs than oncogenes. This is in line with the requirement of loss of the wild-type allele for TSGs. These data indicate that selecting homozygous variants and rare SNPs could indeed enrich for genes or variants linked to a loss of function event. This variant selection strategy is therefore expected to improve associations with functional endpoints.

DISCUSSION

Here we tested whether variant selection strategies that consider allele zygosity and allele frequency help to mark tumor cells with functional defects. To this end we used 29 HNSCC cell lines with confirmed DNA crosslink repair defects as assessed by functional assays. We applied these strategies to the selection of variants in FA/HR pathway genes that govern crosslink repair and found a benefit of selecting homozygous variants and of retaining rare SNPs. The results show that the presence of homozygous variants and rare SNPs in the FA/HR genes is associated with functional outcome, i.e. repair defect, in the cell lines. This highlighted the potential to mark such defects in tumor cells. To demonstrate the value of our approach for exploratory analyses, we extended it to all KEGG pathways. We found that the functional outcome association is strongest in the KEGG Fanconi anemia pathway. This exemplifies the value of the variant selection criteria to identify genes or pathways relevant to a given functional endpoint. Finally, clinical application feasibility was demonstrated in the HNSCC patient tumor analysis. The enrichment of LOH events and homozygous variants in TSGs indicates improved functional association, further highlighting the potential benefit of such variant selection strategies.

Cancer genomics studies often use SNP databases to remove germline variants, but seldom use them to incorporate rare SNPs in their analysis [12]. Guiding variant selection by allele zygosity is even less customary in cancer genomics, even though this approach has successfully been

used to identify pathogenic germline variants in congenital disorders [41,42]. A possible reason why this approach was underused in cancer research, is the complexity of accurately calling homozygous variants caused by LOH in tumors due to intra-tumor heterogeneity, aneuploidy and normal cell admixture [38]. Recently developed algorithms have made progress in meeting this challenge, and the identification of homozygous variants caused by LOH in tumors is now possible [38,39]. Indeed recent studies further point to the importance of considering LOH events and/or homozygosity for marking tumors with HR defects [43,44].

The selection of rare SNPs has been proposed before and was applied previously in order to capture known tumor-associated variants [12]. Since the authors acknowledged the importance of LOH, zygosity and rare SNP criteria have been also applied in attempts to identify genetic DNA repair defects [16]. However, their validity or effectiveness in depicting repair defects has formally never been tested nor confirmed. Here we therefore investigated whether such selection criteria would improve the detection of cell lines with a functional defect or the identification of the relevant genes in repair defected cell lines. In comparison to previous pharmacogenomic projects [5,31,32,45] our cell line panel data provides a unique and reliable model system for studying the effect of variant selection methods on functional outcome association. This is due to a relatively large number of uniformly treated cell lines of the same cancer type and the choice of a robust functional read out, i.e. long-term growth assays to determine MMC IC_{50} values. The commonly used short-term survival assays often reflect a mixture of growth delay and kill. They are also affected by the apoptosis proficiency of a cell line. In contrast, long-term assays reflect the overall repair proficiency, and therefore survival, better. Our chosen drug-doses also ensured that MMC IC_{50} values were reached experimentally rather than estimated by extrapolation. This is a possible improvement to the approach of others [31,32], whose IC_{50} values are discordant [46]. Unfortunately, it was not possible to extend our analysis to the publically available cell line data due to various reasons: lack of publicly available HNSCC cell line data [45], robust MMC response data [32], or DNA sequence information of the canonical FA/HR pathway genes [31]. Two resistant cell lines showed ATP7A variants, while the Hippo pathway depiction was mostly driven by APC and CTNNB1 gene variants and this points to a wnt signaling pathway contribution. A link to platinum drugs has been reported for ATP7A [47]. The validation of the possible role of the wnt signaling pathway in determining MMC resistance is however inhibited by the lack of long-term MMC survival assay data in independent HNSCC cell line panels.

While our analyses showed how the selection of non-synonymous variants, homozygous and rare as SNPs, can identify the genes and pathways responsible for the functional endpoint in question (here the FA/HR pathway for MMC response), it does not show that the selected variants cause the repair defect. This may be the case for a fraction of the selected variants. Others, however, may have simply marked LOH events in the respective genes. As rare SNPs are unlikely to be

present in a homozygous state, selection of homozygosity will therefore mark LOH events and point to gene alterations that are more directly related to the repair defect / functional endpoint. Yet, with respect to the identification of variants with a functional impact, a similar selection procedure did enrich for variants with an annotation in COSMIC or predicted to alter protein function by other algorithms in our previous study [21]. After accounting for the individual stroma component in patient tumor samples, it also enabled a patient outcome association that was not evident with regular selection criteria as shown by our data in Verhagen et al. [21]. Together our data also suggest that variant selection should not solely be guided by pathogenicity criteria (derived from cancer incidence data as by COSMIC annotation) since this results in a lack of hits in our repair defected HNSCC cell line panel. The potential impact on protein function of the selected variants will have to be evaluated in future studies. This is a massive endeavor considering the multitude of variants and questionable in value due to the infrequency of the individual variants. This points to the importance of variant selection strategies that provide an increased link to functional endpoints on a cellular level so to be able to perform valuable outcome association studies.

CONCLUSION

Assuming to be a prerequisite but without proof of concept, homozygocity of DNA repair gene variants has been used to mark tumor samples with potential defects. Here we tested this in the genetic context of tumor cells and show its benefit and association with repair endpoints. The inclusion of rare homozygous SNPs to mark potentially repair defected cell lines further improves this association and highlights their potential role in genomic studies. We conclude that allele zygosity and SNP allele frequency selection criteria can be used to identify FA/HR repair-defected samples and relevant genes.

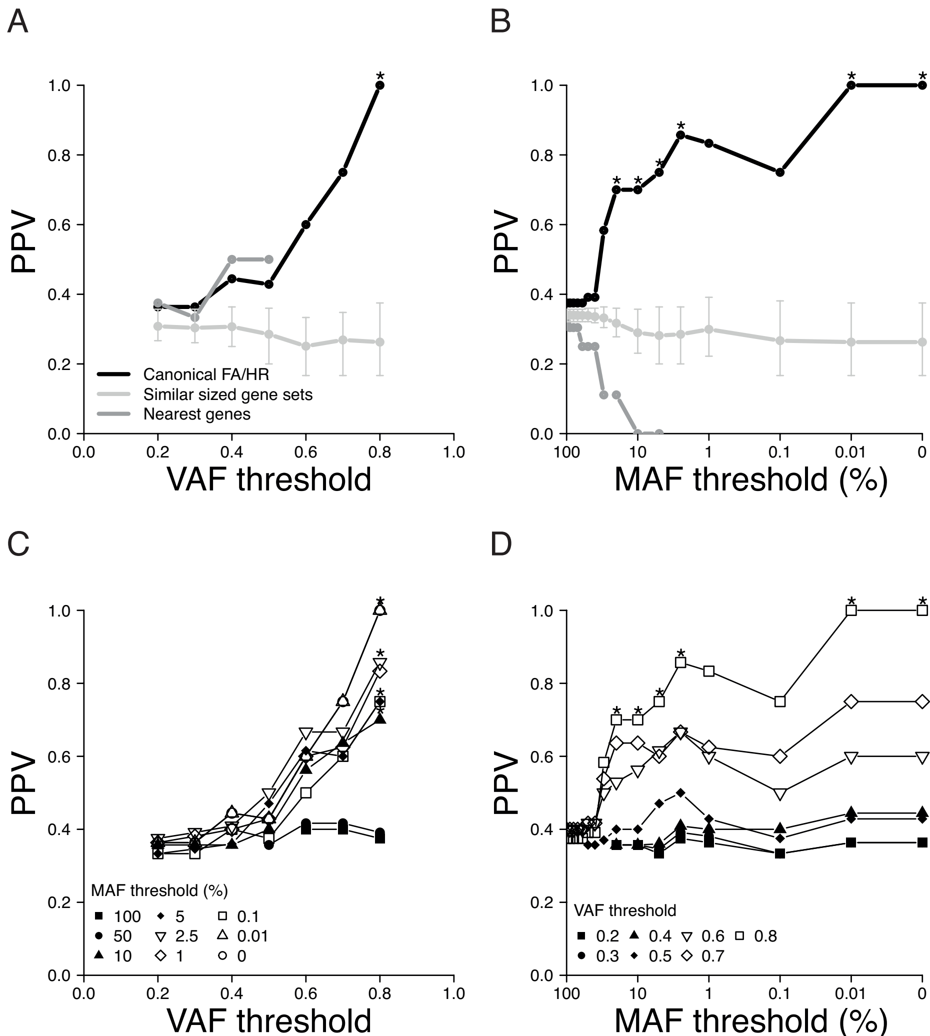

Fig 1. High VAF and low MAF selection criteria improve functional association.

The positive predictive value (PPV) for MMC sensitivity was used to quantify the ability of variants to mark repair defected cell lines, i.e. ten most MMC sensitive, by the different variant selection criteria. Statistical analyses were omitted at sample sizes of a group of less than two and these data points and lines have been excluded in the figures. Asterisks mark a significant association with MMC response. **(A)** PPV values at each VAF threshold that was applied for variant selection are shown. Lines show the results for the canonical FA/HR genes and of the two controls: the "nearest genes" and the 10.000 similar sized randomly grouped gene sets. Error bars delineate the first and third quartile from the median in the latter. **(B)** PPV with progressively decreasing maximum MAF thresholds. Line coloring is identical to Fig 1A. **(C)** Impact of canonical FA/HR gene variant selection filters on PPV when combining multiple maximum MAF thresholds (as indicated in in-figure legend) with increasing minimum VAF thresholds (x-axis). **(D)** Influence of filters on PPVs after applying decreasing maximum MAF thresholds for multiple minimum VAF thresholds for canonical FA/HR gene variant selection as indicated.

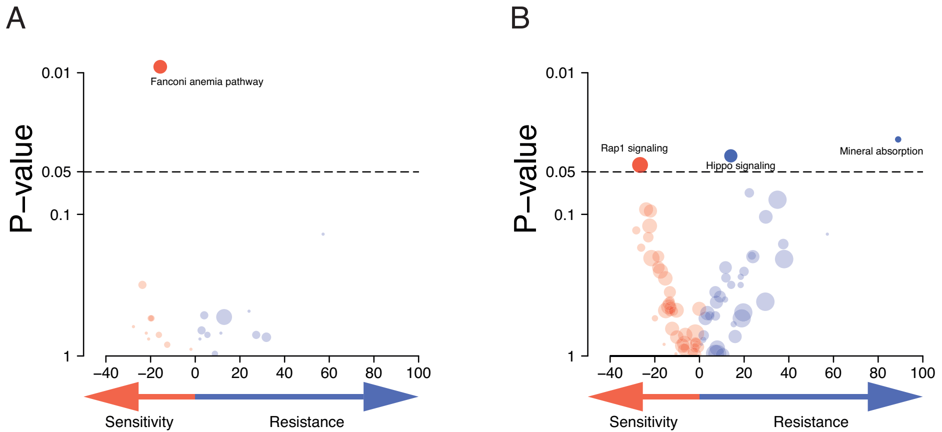

Fig 2. Homozygous and rare SNP variant selection criteria identify the KEGG Fanconi anemia pathway to be strongly associated with MMC response.

Volcano plot showing the significance of the associations between MMC response and 'pathway-mutation' classification in the individual KEGG pathways as determined by the presence of homozygous and rare SNP variants in these pathway genes. The x-axis shows the difference in mean MMC IC_{50} between 'pathway mutated' and 'pathway non-mutated' cell lines. Pathway mutated cell lines are those with one or more variants in any gene of the individual KEGG pathway. The y-axis shows the significance values (Wilcoxon rank-sum test *p*-value) of the difference that were found in the MMC IC_{50} between 'pathway-mutated' and 'non-mutated' cell lines. Dot size is proportional to the number of 'pathway-mutated' cell lines, dot color intensity proportional to significance value. **(A)** Results after applying the variant selection strategy that selects homozygous variants (VAF ≥ 0.8) and includes rare SNPs (MAF ≤ 1%). **(B)** Results of analyses that select variants regardless of zygosity status, including rare SNPs (MAF ≤ 1%).

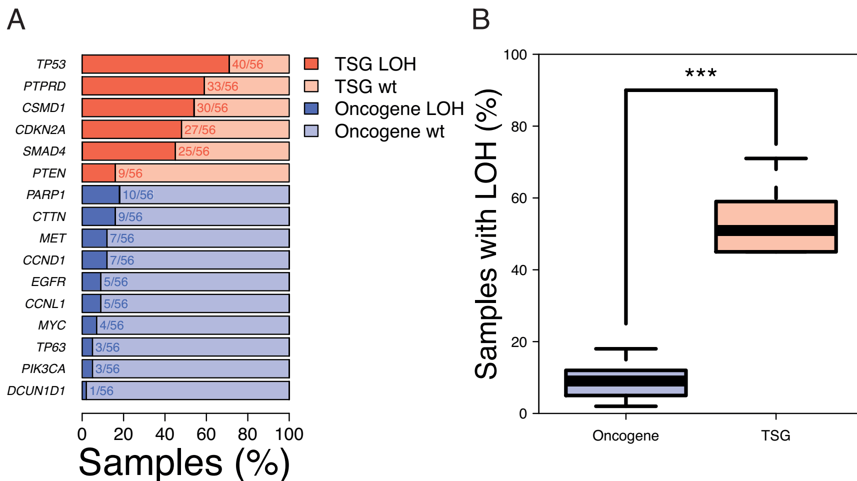

Fig 3. Loss of heterozygosity events are more common in tumor suppressor genes.

(A) The percentage of HNSCC samples with LOH per TSG (dark red) and OG (dark blue). HNSCC TSG and oncogenes (OG) are as reported by Leemans et al. [40]. Light colors represent the percentage of samples without LOH (wt). Fraction of samples with LOH are indicated by numbers and dark colors. **(B)** Boxplot representation of the percentage of HNSCC tumor samples with LOH in any TSGs or OGs as shown in Fig 3A. TSGs are enriched for LOH events ($p < 0.005$).

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SUPPLEMENTARY INFORMATION

The supplementary information referenced in this chapter is freely available online at PLOS ONE (https://doi.org/10.1371/journal.pone.0206632 - Supporting information).

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CHAPTER 4

Comparative genomic analysis of oral versus laryngeal and pharyngeal cancer

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ABSTRACT

Objective

Locally advanced oral squamous cell carcinoma (OSCC) shows lower locoregional control and disease specific survival rates than laryngeal and pharyngeal squamous cell carcinoma (L/P-SCC) after definitive chemoradiotherapy treatment. Despite clinical factors, this can point towards a different tumor biology that could impact chemoradiotherapy response rates. This prompted us to compare the mutational profiles of OSCC with L/P-SCC.

Methods

We performed target capture DNA sequencing on 111 HPV-negative HNSCC samples (NKI dataset), 55 oral and 56 laryngeal/pharyngeal, and identified somatic point mutations and copy number aberrations. We next expanded our analysis with 276 OSCC and 134 L/P-SCC sample data from The Cancer Genome Atlas (TCGA dataset). We focused our analyses on genes that are frequently mutated in HNSCC.

Results

The mutational profiles of OSCC and L/P-SCC showed many similarities. However, OSCC was significantly enriched for *CASP8* (NKI: 15% vs 0%; TCGA: 17% vs 2%) and *HRAS* (TCGA: 10% vs 1%) mutations. *LAMA2* (TCGA: 5% vs 19%) and *NSD1* (TCGA: 7% vs 25%) mutations were enriched in L/P-SCC. Overall, we find that OSCC had fewer somatic point mutations and copy number aberrations than L/P-SCC. Interestingly, L/P-SCC scored higher in mutational and genomic scar signatures associated with homologous recombination DNA repair defects.

Conclusion

Despite showing a similar mutational profile, our comparative genomic analysis revealed distinctive features in OSCC and L/P-SCC. Some of these genes and cellular processes are likely to affect the cellular response to radiation or cisplatin. Genomic characterizations may guide or enable personalized treatment in the future.

INTRODUCTION

Definitive (chemo)radiotherapy (CRT) is a curative treatment option for inoperable, locally advanced oral squamous cell carcinoma (OSCC) [1]. However, it appears that definitive CRT in OSCC does not achieve similarly high locoregional control or disease specific survival rates as in laryngeal and pharyngeal squamous cell carcinoma (L/P-SCC) [2,3]. Current HNSCC treatment guidelines reflect this and L/P-SCC is preferably treated with definitive CRT and OSCC with surgery followed by postoperative radiotherapy with or without chemotherapy (S-PORT). Despite the influence of some clinical factors, the dissimilarity in outcome characteristics could be partly based on a different tumor biology that consequently also impacts CRT response. This led us to question whether the mutational profiles of OSCC and L/P-SCC differ.

Outcomes following S-PORT and CRT are comparable in locally advanced L/P-SCC [3–5], with the exception of T4 L-SCCs [6]. Since CRT preserves the larynx, tongue and tonsils in most patients, it is the preferred treatment for L/P-SCC. In contrast, in locally advanced OSCC, worse outcomes have been reported following CRT in comparison to S-PORT [1,2,7–10]. It should be noted that mainly inoperable OSCCs are treated with CRT, hence impeding any strong conclusion on CRT efficacy in this tumor site. Yet, some studies on operable HNSCC point towards a different CRT response of OSCC and L/PSCC [2,9]. As variation in genetic makeup in OSCC and L/P-SCC could result in altered biology and thereby CRT response, we compared their mutational profiles.

Genomics studies have identified the genes that are frequently mutated in HNSCC. Some focused exclusively on (forms of) OSCC [11–13], others analyzed HNSCC cohorts that comprised multiple subsites as a single entity [14–16]. A markedly different tumor biology was found by comparative genomics studies of HNSCC that focused on the differences between human papillomavirus (HPV)-positive and -negative oropharyngeal tumors [17,18]. Direct comparisons of the mutational profiles of OSCC and L/P-SCC have not yet been performed. Such an analysis could offer explanations for the difference in outcome of OSCC and L/P-SCC following CRT.

We therefore set out to investigate somatic point mutations (SPMs) and copy number aberrations (CNAs) in HPV-negative OSCC and L/P-SCC. We excluded HPV-positive tumors because these have a different genomic make-up, tumor biology and etiology. Specifically, we compared the total and gene-specific rates of SPMs and CNAs between OSCC and L/P-SCC. To this end we employed two datasets. The first dataset consists of targeted DNA sequencing data from 55 OSCC and 56 L/P-SCC tumor samples from our institute (NKI). The second dataset consists of 276 OSCC and 134 L/P-SCC samples, also HPV-negative, from The Cancer Genome Atlas (TCGA).

PATIENTS AND METHODS

Patients

We retrospectively analyzed fresh frozen pretreatment tumor samples from patients treated at our institute between 2001-2010. All patients gave informed consent to have biopsies stored in our tissue bank and used for scientific research. Only biopsies with at least 50% tumor cells as determined on H&E sections were selected for DNA extraction. Samples that were negative for HPV DNA, as determined by p16 staining, targeted DNA sequencing and PCR were included. Together 111 tumor samples ('NKI dataset'), of which 55 were OSCC ($OSCC_{NKI}$) and 56 L/P-SCC ($L/P\text{-}SCC_{NKI}$) were selected. Matched normal samples were unavailable for the majority of tumors and genomic analyses were therefore performed on tumor samples only. From the TCGA we collected data for all available HPV-negative OSCC (n = 276, $OSCC_{TCGA}$) and HPV-negative L/P-SCC (n = 134, $L/P\text{-}SCC_{TCGA}$) samples. NKI and TCGA patient and tumor characteristics are described in Table 1. Whereas the $L/P\text{-}SCC_{NKI}$ dataset consisted of hypo- and oropharyngeal cancers, the $L/P\text{-}SCC_{TCGA}$ dataset consisted mainly of laryngeal tumors (Table 1).

Sequencing and bioinformatics protocol of NKI dataset

Details of the sequencing and bioinformatics protocols applied in the NKI dataset are specified in the Supplementary Methods [19–27]. In short, we performed target capture DNA sequencing of 556 human genes (Supplementary Table 1). HPV gene baits, to capture HPV DNA in the samples, were included in order to determine the HPV status. We removed DNA sequence variants that were in any of three public SNP databases [25–27] and classified the remaining variants as SPMs (listed in Supplementary Table 2). Homozygous deletions and focal amplifications were detected using the R package PureCN [22].

The Cancer Genome Atlas data

We collected open access clinical, SPM and CNA data for the TCGA samples from the most recent available Firehose run (28-12-2016). SPMs of TCGA were detected by comparing whole exome sequencing data of tumors with their matched normal samples. For analyses of individual genes non-silent SPMs were selected. We included silent mutations for analyses on the total number of SPMs and the determination of transitions and transversions (TiTvs) rates. TiTvs were generated with the GenVisR package [28]. Through assessing the relative contribution of single-nucleotide polymorphism in a sample, a copy number profile can be generated using SNP array data [29,30]. CNAs were detected based on whole genome SNP6 arrays. These were available for the TCGA dataset [29,30]. From the gene level data, we selected CNAs that exceeded the chromosome arm aberrations in each sample. In TCGA data, these values are typically regarded as homozygous deletions and focal amplifications. We considered these CNAs to correspond best to those of the NKI dataset, because they both represent high amplitude CNAs. Furthermore, we

performed an analysis to identify regions that were significantly amplified or deleted across all TCGA HNSCC samples. These were identified with the GISTIC2 algorithm [31] and are part of the open access data. Genomic scar signature scores were available for 141/276 OSCC and 76/134 L/P-SCC samples from the supplementary data of [32]. The codes to reproduce all analyses on TCGA data are available at https://github.com/dvossen/OSCC-versus-LPSCC.

Frequently mutated genes in HNSCC

Mutational profiles of OSCC and L/P-SCC tumors are based on a gene set of genes that are frequently mutated in HNSCC, as identified in [33]. These consisted of 168 genes with frequent SPMs (‘$genes_{SPM}$’, Supplementary Table 3) and 25 genes with frequent CNAs (‘$genes_{CNA}$’, Supplementary Table 4). To warrant a sufficient high statistical power, we limited our analyses to these genes. In addition, events will have to be frequent to explain a differential CRT response. In [33], ‘$genes_{SPM}$’ were identified by algorithms that select genes with more SPMs than expected by chance given various background mutation rates and processes. The ‘$genes_{CNA}$’ came from regions frequently affected by focal CNAs in HNSCC [33] and contain 25 genes that are annotated in the Cancer Gene Census [34] (Supplementary Table 4).The NKI targeted sequencing efforts captured 27 out of these 168 ‘$genes_{SPM}$’ and 11 of the 25 ‘$genes_{CNA}$’ (Supplementary Table 3 and 4). NKI dataset analyses are based on this subset of genes and TCGA data on all ‘$genes_{SPM}$’ and ‘$genes_{CNA}$’.

Statistical methods

Correlation coefficients refer to Spearman’s rank correlation coefficient. We used Fisher’s exact test to compare proportions between OSCC and L/P-SCC and the Wilcoxon rank-sum test to compare numerical variables. For the tests on the $genes_{SPM}$ and $genes_{CNA}$ we controlled the false discovery rate at 0.10 by correcting for multiple hypothesis testing with the Benjamini and Hochberg method. The corrected *p*-values are reported as Q-values. Error bars on proportions report the 95% confidence interval (Wilson score interval). We used a binomial mixed model to compare the proportion of each TiTv in OSCC and L/P-SCC, with subsite as a fixed and sample as a random effect. We used the log-rank test and Cox proportional hazards model to test for associations between clinical or genetic features and overall survival. All statistical analyses were performed in the R environment for statistical computing.

RESULTS

Mutational profiles: major similarities and minor differences between subsites

We first determined the mutational profiles of the NKI tumor samples in terms of somatic point mutations (SPMs) and copy number aberrations (CNAs), and compared them to the TCGA. We restricted our analysis to gene sets with genes that were previously found to have frequent SPMs (‘$genes_{SPM}$’) and CNAs (‘$genes_{CNA}$’) in the TCGA HNSCC data (see Methods). Figure 1A gives an overview of the frequently mutated genes in all samples. We then calculated the percentage of samples that carry a SPM in each individual $gene_{SPM}$ and a CNA of each $gene_{CNA}$, within $OSCC_{NKI}$, $OSCC_{TCGA}$, $P\text{-}SCC_{NKI}$ and $L/P\text{-}SCC_{TCGA}$. These revealed many similarities across datasets and subsites, with frequent SPMs in *TP53*, *CDKN2A* and *PIK3CA* and CNAs in *CCND1*, *CDKN2A* and *EGFR* (Figure 1B and 1C). When clustering on affected $genes_{SPM}$, or $genes_{CNA}$ OSCC from different datasets clustered with each other (Supplementary Figure 1). Overall similarities are further supported by the high correlations between the datasets and across subsites as illustrated in Supplementary Figure 2 (ρ range 0.71-0.85).

In summary, the mutational profiles are remarkably similar. However, there are differences both between datasets and subsites. Therefore, to identify differences between subsites while excluding dataset bias, we compared the mutational profiles of subsites within datasets.

OSCCs and L/P-SCCs have distinct patterns of somatic point mutation affected genes

To expose the differences between OSCC and L/P-SCC, we next compared the percentage of samples with SPMs in the $gene_{SPM}$ set. After correcting for multiple hypothesis testing, significance was reached for only one gene in the NKI and for four in the TCGA dataset (Figure 2A). *CASP8* mutations occurred almost exclusively in OSCC in both the NKI (15% vs 0%; $Q < 0.1$) and TCGA datasets (17% vs 2%; $Q < 0.001$). *HRAS* mutations followed this trend, which reached significance in the TCGA dataset (10% vs 1%; $Q < 0.01$). In contrast, *LAMA2* (5% vs 19%; $Q < 0.01$) and *NSD1* mutations (7% vs 25%; $Q < 0.001$) occurred less frequently in OSCC than L/P-SCC. *LAMA2* and *NSD1* were not sequenced in the NKI dataset.

Next, we compared the total number of SPMs (including silent mutations, see Methods) per sample between subsites. This was performed in the TCGA dataset only, since the total number of mutations was too small in the NKI dataset due to the limited targeted sequencing (556 genes). We found that L/P-SCC had significantly more mutations per sample than OSCC (median 191 vs 146 per sample; $p < 0.001$) (Figure 2B). We then investigated the distribution of the different possible transitions and transversions (TiTvs) in the SPMs. Each individual TiTv was more frequent in L/P-SCC samples (Supplementary Figure 3A). However, proportionally, G->A/C->T transitions were more common ($p < 0.001$) and G->T/C->A transversions less common ($p <$

0.001) in OSCC (Supplementary Figure 3B). Prompted by these increased numbers and altered mutation spectrum, we assessed which of 30 COSMIC mutational signatures (http://cancer.sanger.ac.uk/cosmic/signatures, 18-12-2017) was present in each tumor sample in order to infer the cause of these differences (Supplementary Methods). We found five signatures with a significant difference in the prevalence of these signatures (Figure 2C). Signatures 1 and 15, associated with age and DNA mismatch repair respectively, are more prevalent in OSCC. L/P-SCC, in contrast, are characterized by a prevalence of signatures 3, 4 and 24, that are associated with homologous recombination (HR) deficiency, smoking and aflatoxin exposure respectively. The prevalence of the age and smoking associated signatures is consistent with the relative contribution of smokers and younger patients within the different patient populations (Table 1).

In summary, OSCC is characterized by frequent *CASP8* and *HRAS* mutations and relatively infrequent *LAMA2* and *NSD1* mutations. The mutational burden is higher in L/P-SCC and they show more frequently signs of HR DNA repair defects.

Common copy number aberrations occur less frequently in OSCCs

To characterize larger genomic aberrations, we next compared the percentage of OSCC and L/P-SCC samples with CNAs in the $\text{genes}_{\text{CNA}}$. The occurrence of CNAs was significantly lower in OSCC compared to L/P-SCC for *CCND1* and *TP63*. *CCND1* was less frequently amplified in OSCC than L/P-SCC in both the NKI (24% vs 70%; $Q < 0.001$) and TCGA datasets (23% vs 37%; $Q < 0.1$) (Figure 2D). *TP63* (14% vs 35%; $Q < 0.001$) was also less frequently amplified in OSCC than L/P-SCC in the TCGA dataset. The trend was the same in the NKI dataset, but did not reach significance. Interestingly, both genes were less frequently amplified in OSCC than L/P-SCC. This led us to question whether OSCCs have in general fewer CNAs than L/P-SCCs.

First, for each sample, we considered the total number of CNAs amongst the $\text{genes}_{\text{CNA}}$ set. We compared OSCC with L/P-SCC samples and found that OSCC samples have fewer CNAs in both the NKI ($p < 0.001$) and TCGA ($p < 0.001$) datasets (Supplementary Table 5). Others have shown that *CASP8*-mutated HNSCC harbor fewer CNAs than *CASP8*-wildtype HNSCC [11]. Given the enrichment for *CASP8* mutations in OSCC, we also compared the frequency of CNAs while excluding *CASP8*-mutated samples. We still found fewer CNAs in OSCC in both, the NKI ($p < 0.01$) and TCGA ($p < 0.05$) datasets (Supplementary Table 5). Finally, per sample, we considered the total number of CNAs amongst regions that are significantly often amplified or deleted across all TCGA HNSCC samples. This analysis was performed on whole genome copy number data that was possible in the TCGA data set. OSCC harbored fewer regions of amplification and deletion than L/P-SCC, both including ($p < 0.001$) and excluding ($p < 0.01$) *CASP8*-mutated samples (Figure 3A and 3B). As *CASP8* and *HRAS* mutations frequently co-occur in OSCC [11] (in $\text{OSCC}_{\text{TCGA}}$ 40% of *CASP*-mutated versus 4% of *CASP*-wildtype tumors harbored *HRAS* mutations, $p < 0.001$),

we further stratified by *HRAS* mutation status as well. Also *HRAS*-wildtype OSCC showed fewer regions of amplification and deletion than their *HRAS*-wildtype L/P-SCC counterparts (Figure 3C).

Taken together, these copy number data show that OSCC harbor fewer CNAs and in particular less frequent amplification of *CCND1* and *TP63* than L/P-SCC.

OSCCs have fewer genomic scars

Chromosomal instability processes may underlie the observed increase in CNAs in L/P-SCC. HR deficiency can cause chromosomal instability that results in gross CNAs [35], often referred to as 'genomic scars'. Three SNP array-based genomic scar signatures were applied to the TCGA HNSCC dataset in a recent study [32]. These signatures quantify the level of a particular pattern of genomic scarring and each signature is associated with HR deficiency (i.e. *BRCA1* or *BRCA2* mutations) or markers thereof (cisplatin sensitivity). The signatures are: Number of telomeric Allelic Imbalances (NtAI) [36], Large Scale Transition (LST) [37] and Homologous Recombination Deficiency (HRD) score [38]. Here we compared signature scores between OSCC and L/P-SCC, in order to probe for possible HR deficiency differences. We find that OSCCs had significantly lower genomic scar scores than L/P-SCCs. This was true for all tested signatures (Figure 4A). Stratifying subsites according to *CASP8* mutation status revealed a consistent pattern across all three signatures: *CASP8*-mutated OSCC had the lowest score, then *CASP8*-wild type OSCC and finally *CASP8*-wild type L/P-SCC (Figure 4B). There were no *CASP8*-mutated L/P-SCC samples with available signatures scores in this dataset. The pattern was similar when stratifying by *HRAS* mutation status (Figure 4C).

To further quantify potential HR deficiency across subsites, we split samples at the median HRD score following the example of Abkevich et al. [38]. The proportion of samples with a high HRD score was substantially higher in the L/P-SCCs than OSCCs, regardless whether *CASP8*-mutated tumors were included (76% vs 45%; $p < 0.001$) or excluded (76% vs 50%; $p < 0.001$). These results are in line with our observation that L/P-SCCs have a higher prevalence of an HR deficiency associated mutational signature (Figure 2C). Samples with presence of the HR deficiency associated mutational signature had higher genomic scar signature scores, thereby further sustaining this finding (Supplementary Figure 4).

In a previous study (manuscript submitted), we found functional HR and Fanconi anemia (FA) pathway defects in a panel of HNSCC cell lines that were accompanied by FA/HR gene SPMs. As SPMs in HR/FA genes (Supplementary Table 6) may have caused the observed HR deficiencies, we tested for an association between such mutations and the signature scores in all samples with available scar signature scores. HR/FA-mutated tumor samples had significantly higher NtAI and LST scores (Figure 5A). HRD scores were, however, similar. This pattern was also still

present when analyzing OSCC and L/P-SCC separately (Figure 5B and 5C), although not always significant, possibly due to the smaller sample sizes. Finally, the proportion of HR/FA-mutated OSCCs was lower than that of L/P-SCC in the TCGA ($p < 0.05$), but this could not be confirmed in the smaller NKI dataset (Supplementary Figure 5).

Overall, our results indicate that OSCC harbor less HR defects as determined by genomic scar signatures. This holds true when excluding the CNA-silent subgroup of *CASP8*-mutated tumors, enriched in OSCCs. Thus, a lower incidence of HR deficiency in OSCC than L/P-SCC might underlie the lower incidence of CNAs in OSCC.

Associations of mutational features with clinical outcomes

OSCC, compared to L/P-SCC, showed largely similar but also distinctly different mutational features. Contemporary OSCC treatment regimens prioritize surgery over CRT, partly due to the absence of a pronounced CRT response in OSCC in the past. Thus, this resulted in a lack of CRT-treated OSCC patients available for genomic CRT response association studies. In an attempt to investigate the potential clinical relevance of the here observed biology characteristic to L/P-SCC or OSCC, i.e. genetic aberrations, we explored alternative data bases. Primarily set up for genomic studies, the TCGA data contains only surgically treated cases and clinical data are incomplete. To test adequacy, we first analyzed whether the two HNSCC datasets display the known impact of strong clinical factors (T-stage, N-stage and age) on overall survival (Supplementary Figure 6). As the OSCC clinical data behaved as expected, we next tested whether the individual mutational features are associated with clinical outcome (Supplementary Table 7). We find that mutational burden, NtAI score and *CASP8* mutations are connected to poor prognosis in this patient group (Figure 6A-C). To further deduce a role in platinum-based treatment responses, we investigated the relevance of the genomic alterations in the TCGA ovarian cancer dataset (Supplementary Figure 6 and Supplementary Table 8). These patients received adjuvant platinum-based chemotherapy [39]. Here we observe that mutational burden, NtAI and LST are associated with a better overall survival (Figure 6D-F and Supplementary Table 8), suggesting that patients with tumors with such characteristics may benefit from platinum-based treatments.

Together, these observations could point to a favorable platinum response of tumors with the genetic characteristics of L/P-SCC, in particular repair-defect associated signatures and scars. They are less frequent in OSCC, however relevant to outcome if present. This might explain the lack of a pronounced CRT response in this tumor site.

DISCUSSION

Prompted by clinical indications that OSCC may respond differently to CRT than L/P-SCC, we compared the mutational profiles of OSCC and L/P-SCC. We identified six genes that are more frequently mutated in either OSCC or L/P-SCC. We find that OSCC has fewer SPMs and CNAs than L/P-SCC. Important in the context of CRT response, the subsites score differently in mutation and genomic scar signatures associated with HR deficiency.

Our analyses show that OSCC scores lower than L/P-SCC on three HR deficiency associated scar signatures. These signatures scores were positively associated with SPMs in HR/FA genes. These results thus strongly suggest that L/P-SCC harbor HR defects, and that those are less common in OSCC. It should be noted that these signatures were developed for breast and ovarian cancer. Two of these signatures are associated with mutations in the HR genes *BRCA1* and *BRCA2*, in basal-like breast cancer [37] and ovarian tumors [38]. The third signature is associated with cisplatin sensitivity in breast cancer cell lines [36]. In our analysis in HNSCC, we found, however, overall a good interrelation between genomic scar signatures, COSMIC HR signatures and the presence of SPMs in FA/HR genes, together supporting biological signification of the scoring. Importantly, cells and tumors with HR defects are particularly sensitive to platinum-based chemotherapeutics such as cisplatin [35]. Clinical association studies based on TCGA data should be considered with caution. In addition, TCGA patients all underwent surgery, hampering a comparison to definite CRT. Yet, our observations do illustrate a potential link between the genetic characteristics related to HR defects and the reported superior CRT response in L/P-SCC. Indicating relevance in HNSCC, they depicted poor prognosis in the OSCC patient dataset. A prolonged survival was evident in patients with ovarian cancers that showed high mutational burden or signs of HR defects. Treated with platinum-based adjuvant chemotherapy [39], this could point to a favorable treatment setting for such HR-affected tumors. With a decreased incidence in OSCC, this would be therefore consistent with an overall decrease in the CRT response. Taken together, our findings could therefore provide the basis for a differential CRT response and explain why the reported outcomes of L/P-SCC following platinum-based CRT may be better than those of OSCC [2,7,8,10].

The HNSCC $genes_{SPM}$ and $genes_{CNA}$ sets where originally identified in the TCGA dataset [33]. The TCGA HNSCC dataset however mostly consists of OSCC tumor samples (roughly two thirds), thereby possibly causing some bias in the gene set selection. Genes which were frequently mutated in OSCC were therefore well captured. However, we acknowledge that due to the smaller proportion of L/P-SCC samples in the TCGA dataset, genes that are frequently mutated in L/P-SCC but not OSCC might have remained undetected. Yet, while also including our NKI

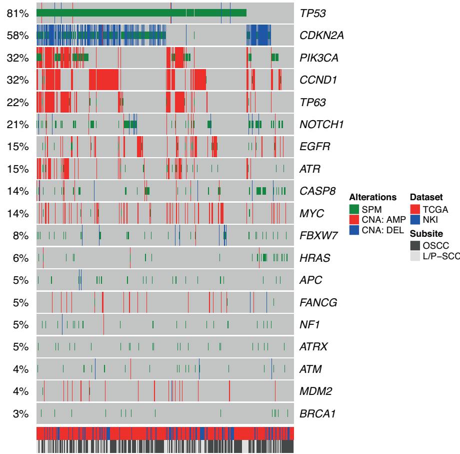
data set, we were able to assess well whether or not these genes$_{SPM}$ and genes$_{CNA}$ were also frequently mutated in L/P-SCC.

The results of our analysis show that OSCCs are enriched for *CASP8* and *HRAS* mutations and are consistent with other studies that identified a subset of OSCCs characterized by frequent *CASP8* mutations co-occurring with *HRAS* mutations and infrequent CNAs [11,14]. One of these studies found that *CASP8*-mutated tumors were more tumorigenic and larger in an orthotopic model of OSCC [11]. Another study demonstrated that *CASP8* mutations promote migration and invasion in HNSCC cell lines and tumor growth in mouse xenografts [40]. These experimental results seem in contradiction with clinical data, which indicate that HNSCC with infrequent CNAs have a relatively good outcome [14,41]. *CASP8* has a role in the extrinsic apoptotic pathway that may also influence treatment resistance [42,43]. Cisplatin- and radiotherapy-induced apoptosis signals have been correlated to tumor response in some cancer types [44,45]. Whether inactivating *CASP8* mutations in OSCC [40] could contribute to a poor CRT response, will have to be evaluated in future studies. The finding that *CASP8*-mutated OSCCs harbor fewer CNAs than other OSCC is supported both by our and other's data [11,14]. Our results further demonstrate that OSCC is a subsite with fewer CNAs even when *CASP8* wild-type. Another finding of this study was enrichment of *LAMA2* and *NSD1* mutations in L/P-SCCs. *NSD1* encodes a histone methyltransferase. An epigenome deregulation study conducted on TCGA data confirms the enrichment of *NSD1* mutations in laryngeal carcinomas [46]. The enrichment of *LAMA2* mutations in L/P-SCC has not yet been reported to our knowledge. *LAMA2* (encoding a subunit of laminin) germline loss has been reported in neurofibromatosis type 1 associated tumors [47] and hypermethylation in colorectal carcinoma [48]. As positive selection of *LAMA2* mutations suggests functional involvement of this gene, it raises the question why this is in particular the case in L/P-SCC. Future research may answer the subsite-specific role of these mutated genes.

CONCLUSION

There are genetic differences between OSCC and L/P-SCC with respect to several genes that are frequently mutated. COSMIC signatures, genomic scars and frequent CNAs reveal possible differences in HR deficiency. This reflects a different biology that could provoke an altered CRT response. Our findings could stimulate future investigations into more individualized treatment protocols for different subsites of head and neck cancer.

A



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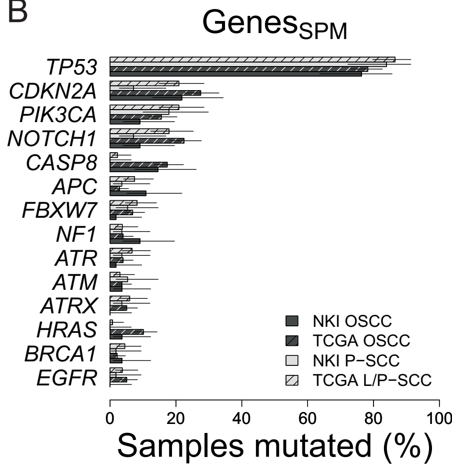

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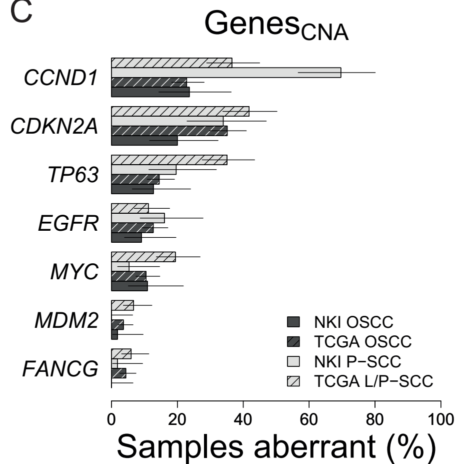

Figure 1. Frequently mutated genes in the NKI and TCGA dataset.

(A) An overview of the mutations found in the most frequently mutated genes$_{SPM}$ and genes$_{CNA}$. Genes correspond to those in (B) and (C). **(B)** Genes$_{SPM}$ and their frequency of somatic point mutations in the NKI and TCGA datasets. Shown are the genes$_{SPM}$ that have a somatic point mutation in at least 5% of samples in at least one dataset, plus *BRCA1*. Errors bars show the 95% confidence interval of the mean. **(C)** Genes$_{CNA}$ and their frequency of copy number aberration in the NKI and TCGA datasets. Shown are the genes$_{CNA}$ that have a copy number aberration in at least 5% of samples in at least one datasets. Error bars show the 95% confidence interval around the mean.

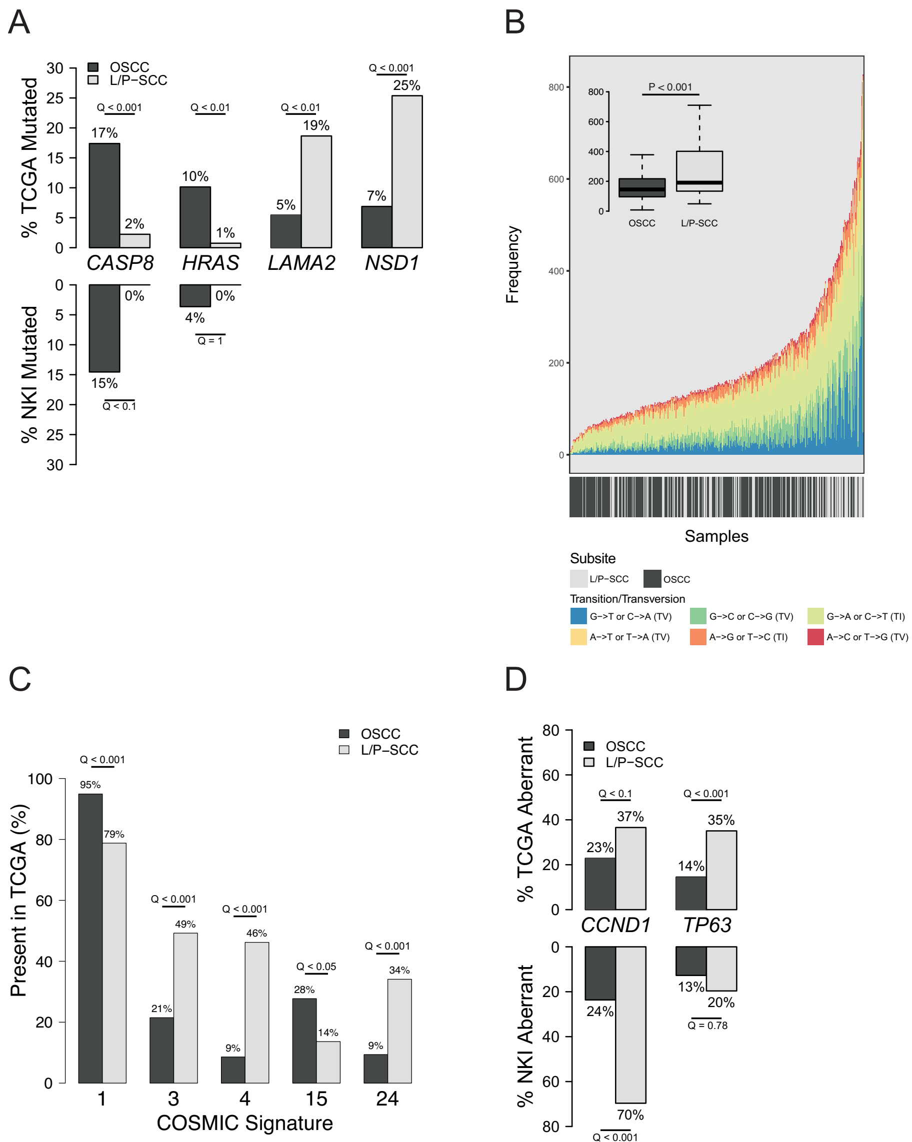

Figure 2. Genetic mutations that occur with significant different frequencies between HNSCC subsites.

(A) Genes$_{SPM}$ with a significant different frequency of somatic point mutations between oral squamous cell carcinomas (OSCC) and laryngeal and pharyngeal squamous cell carcinomas (L/P-SCC). Bars show the frequency of somatic point mutations in these HNSCC subsites, in the TCGA (top) and NKI (bottom) dataset. **(B)** Frequency of transitions and transversions (TiTvs) (y-axis) in each TCGA sample (x-axis). One sample without somatic mutations and four samples with more than 1,000 somatic mutations are not shown. Inlay boxplot compares the distribution of total number of TiTvs per sample between subsites (outliers not shown, but included in statistical testing). **(C)** COSMIC mutational signatures that were present in significantly higher proportion of OSCC or L/P-SCC. **(D)** Genes$_{CNA}$ with significant different frequency of copy number aberrations between OSCC and L/P-SCC.

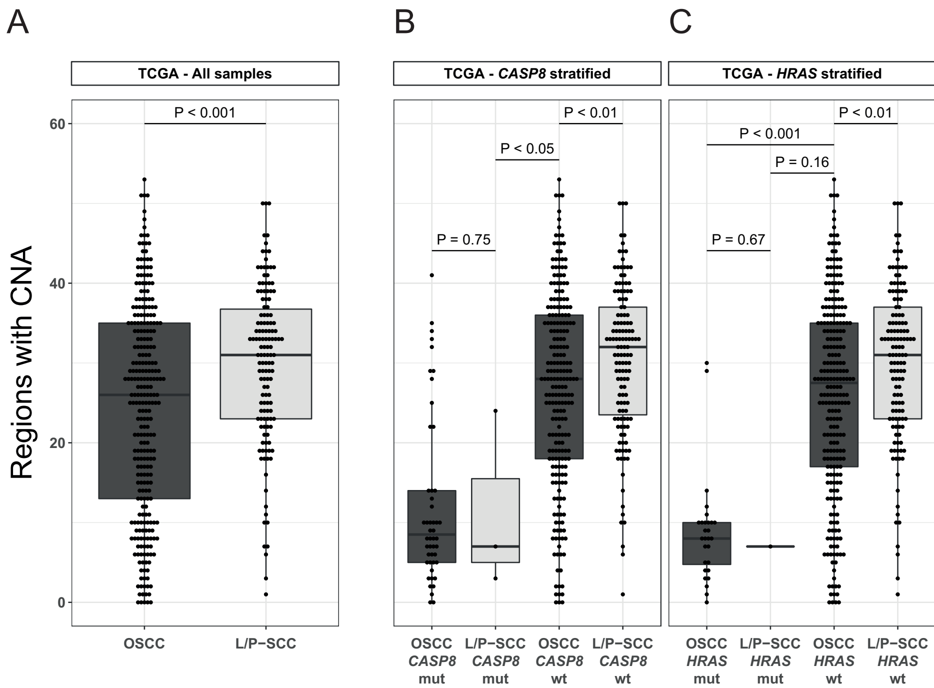

Figure 3. OSCC has fewer copy number aberrations.

(A) Boxplot of the number of regions with focal copy number aberration (CNA) in all oral squamous cell carcinoma (OSCC) and laryngeal and pharyngeal squamous cell carcinoma (L/P-SCC) samples from the TCGA dataset. **(B)** Same as (A), but subsites are stratified according to *CASP8* mutation status. **(C)** Same as (A), but subsites are stratified according to *HRAS* mutation status.

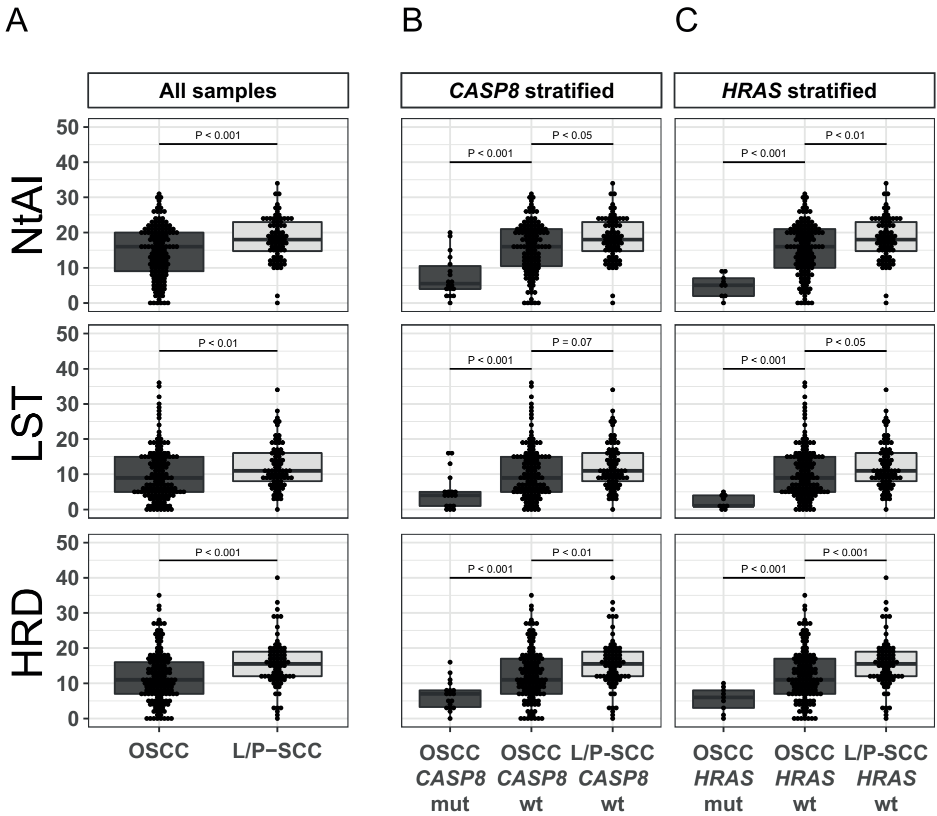

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Figure 4. OSCC has fewer genomic scars.

(A) Boxplot of genomic scar signature scores in 141 oral squamous cell carcinoma (OSCC) and 76 laryngeal and pharyngeal squamous cell carcinoma (L/P-SCC) samples from the TCGA dataset. The signatures are: number of telomeric Allelic Imbalances (NtAI), Large Scale Transition (LST), Homologous Recombination Deficiency (HRD) score. **(B)** Same as (A), but subsites are stratified according to *CASP8* mutation status. **(C)** Same as (A), but subsites are stratified according to *HRAS* mutation status.

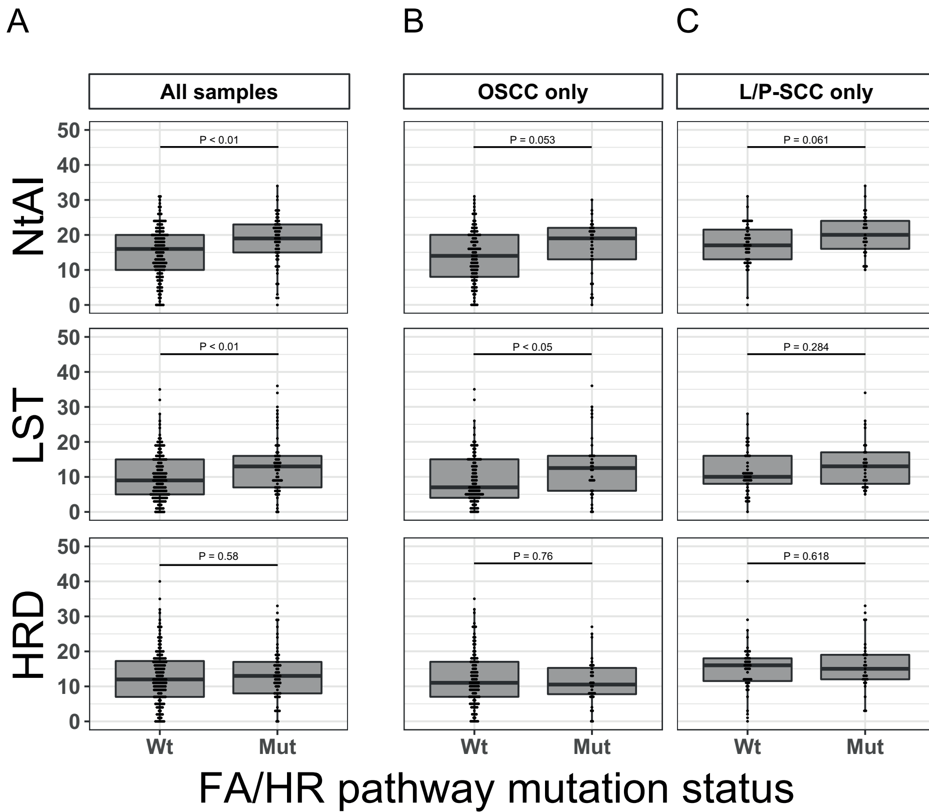

Figure 5. Somatic point mutations in Homologous recombination and Fanconi anemia genes are associated with genomic scar signature scores.

(A) Genomic scar signature scores in 141 oral squamous cell carcinoma (OSCC) and 76 laryngeal and pharyngeal squamous cell carcinoma (L/P-SCC) samples from the TCGA dataset. Boxplots compare score signatures of samples with a somatic mutation in any of the HR/FA genes (Mut) and those wildtype for all HR/FA genes (Wt). **(B)** Same as (A), but only showing OSCC samples. **(C)** Same as (A), but only showing L/P-SCC samples.

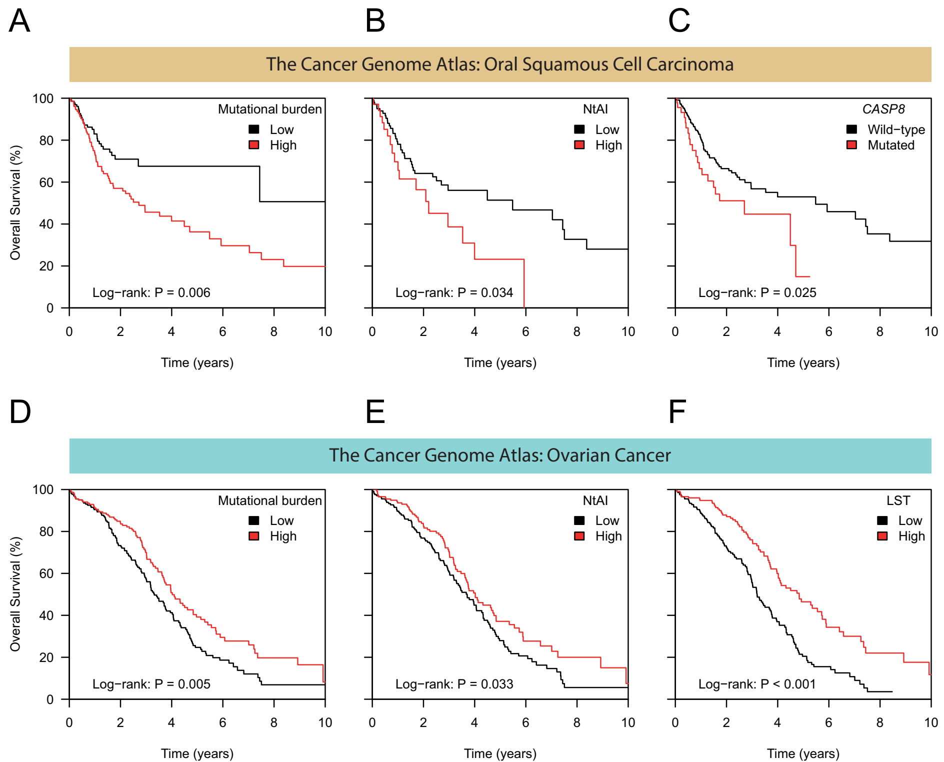

Figure 6. Illustration of a potential clinical role of the OSCC or L/P-SCC specific mutational features.

Overall survival (OS) of TCGA OSCC patients stratified by **(A)** the total number of SPMs per sample ('mutational burden') (median split), **(B)** NtAI score (third quartile split) and **(C)** *CASP8* SPM mutation status. Mutational burden and NtAI score are continuous variables, and as such were associated with OS in Cox proportional hazards models ($p = 0.01$ and $p = 0.005$ in univariate models, respectively). Subsequently, these variables were split for illustration purposes, and the corresponding log-rank test *p*-values are shown in-figure. Similarly, in the TCGA ovarian cancer dataset **(D)** mutational burden, **(E)** NtAI score and **(F)** LST score were each associated with OS as a continuous variable ($p = 0.013$, $p = 0.029$ and $p < 0.001$ in univariable Cox models, respectively). Each was subsequently median split for illustration purposes. Supplementary Tables 7 and 8 contain the hazard ratios and *p*-values of all uni- and multivariable Cox models for the TCGA OSCC and ovarian cancer datasets, respectively.

Table 1. Patient and tumor characteristics.

	$OSCC_{NKI}$ (n = 55)		$P\text{-}SCC_{NKI}$ (n = 56)		$OSCC_{TCGA}$ (n = 276)		$L/P\text{-}SCC_{TCGA}$ (n = 134)	
Variable	N	%	N	%	N	%	N	%
Age								
Mean (SD)	63 (12)		60(9)		62 (14)		61 (9)	
Median (range)	64 (43-93)		58 (37-78)		62 (19-90)		61 (38-85)	
Gender								
Male	40	73	39	70	179	65	108	81
Female	15	27	17	30	97	35	26	19
Primary site								
Hypopharynx	0	0	28	50	0	0	5	4
Larynx	0	0	0	0	0	0	105	78
Oral cavity	55	100	0	0	276	100	0	0
Oropharynx	0	0	28	50	0	0	24	18
T stage[a]								
T0	0	0	0	0	0	0	1	1
T1	9	16	1	2	27	10	10	7
T2	27	49	8	14	87	32	15	11
T3	7	13	24	43	56	20	32	24
T4	12	22	23	41	92	33	59	44
Tx	0	0	0	0	7	3	12	9
Missing	0	0	0	0	7	3	5	4
N stage[a]								
N0	28	51	9	16	109	39	46	34
N1	9	16	7	13	45	16	15	11
N2	17	31	35	63	83	30	46	34
N3	1	2	5	9	2	1	3	2
Nx	0	0	0	0	29	11	19	14
Missing	0	0	0	0	8	3	5	4
Smoker								
Current or former	35	64	50	89	191	69	119	89
Never	12	22	1	2	78	28	12	9
Unknown	8	15	5	9	7	3	3	2

Table 1: Continued

	$OSCC_{NKI}$ (n = 55)		$P\text{-}SCC_{NKI}$ (n = 56)		$OSCC_{TCGA}$ (n = 276)		$L/P\text{-}SCC_{TCGA}$ (n = 134)	
Variable	N	%	N	%	N	%	N	%
Alcohol consumption								
Yes	30	55	51	91	78	28	45	34
No	18	33	2	4	37	13	7	5
Unknown	7	13	3	5	161	58	82	61

[a] Pathologic stages are reported for $OSCC_{NKI}$, $OSCC_{TCGA}$ and $L/P\text{-}SCC_{TCGA}$, clinical stages for $P\text{-}SCC_{NKI}$.

ABBREVIATIONS

CNAs (copy number aberrations), CRT (chemoradiotherapy), FA (Fanconi anemia), HPV (human papillomavirus), HR (Homologous recombination), HRD (Homologous Recombination Deficiency), L/P-SCC (laryngeal and pharyngeal squamous cell carcinoma), LST (Large Scale Transition), NtAI (number of telomeric Allelic Imbalances), OSCC (oral squamous cell carcinoma), SPMs (somatic point mutations), S-PORT(surgery followed by postoperative radiotherapy with or without chemotherapy), TCGA (The Cancer Genome Atlas), TiTvs (transitions and transversions).

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SUPPLEMENTARY INFORMATION

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CHAPTER 5

Genetic Factors Associated with a Poor Outcome in Head and Neck Cancer Patients Receiving Definitive Chemoradiotherapy

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ABSTRACT

About half of advanced stage head and neck squamous cell carcinoma (HNSCC) patients can be cured by chemoradiotherapy. Patient outcome may be partially determined by the genetic alterations in HNSCC, rendering these alterations promising candidate prognostic factors and/or therapeutic targets. However, their relevance in patient outcome prognosis remains to be assessed in patients that receive standard-of-care chemoradiotherapy. We therefore tested whether frequent genetic alterations were associated with progression free survival (PFS) in advanced stage HNSCC patients who were uniformly treated with definitive platinum-based chemoradiotherapy. To this end, we performed targeted DNA sequencing on frozen pre-treatment tumor biopsy material from 77 patients with advanced stage oro- and hypopharyngeal carcinoma. This provided somatic point mutation and copy number aberration data of 556 genes. The most frequently mutated genes, *TP53* (62%), *CCND1* (51%), *CDKN2A* (30%) and *PIK3CA* (21%), were not associated with PFS. However, co-occurring *CCND1* and *CDKN2A* mutations were associated with short PFS (HR 2.24, $p = 0.028$) in HPV-negative tumors. Furthermore, tumor mutational burden (sum of somatic point mutations) showed a trend towards decreased PFS (HR 1.9, $p = 0.089$), and chromosomal instability (CIN) was associated with shorter PFS (HR 2.3, $p = 0.023$), independent of HPV status. Our results show that tumor mutational burden, CIN markers, and co-occurring *CCND1* and *CDKN2A* mutations are associated with chemoradiotherapy outcomes in advanced stage oro- and hypopharyngeal HNSCC patients, thereby highlighting their prognostic potential. Given their poor prognosis association and link to biological targets, they may also identify patients for novel targeted therapies and immunotherapies.

INTRODUCTION

Advanced stage head and neck squamous cell carcinoma (HNSCC) has a poor prognosis. Chemoradiotherapy cures about half of all patients with advanced HNSCC. Allowing the preservation of organ function, it has become the preferred treatment option for these cancers [1]. Patients with a poor prognosis under such standard treatment may be eligible for increased surveillance and/or alternative treatments if they could be identified upfront. However, the current TNM staging [2] based prognosis is very limited in this regard and novel prognostic biomarkers are therefore urgently needed.

Tumor growth and treatment responses are largely determined by tumor intrinsic genetics. Genomic features therefore hold the promise of providing potential treatment response biomarkers. Frequent alterations are of particular interest for this purpose. Previous studies identified commonly mutated genes through large comprehensive DNA sequencing efforts and genetic analyses, thereby revealing the “driver genes” in HNSCC [3–5]. Their prognostic value is currently under investigation [6–12]. Pairs of co-occurring mutated genes can also hold prognostic information [13], and this can point to a functional link between the events. This can also apply across different types of genetic features in tumors; e.g., mutations in mismatch repair genes are linked to a high mutational burden [14] and homologous recombination repair gene mutations to overall gene copy number alterations (CNAs) [15].

DNA crosslink repair defect analyses in a large panel of patient-derived HNSCC cell lines revealed that HNSCC are frequently characterized by DNA repair (DR) defects [16]. Potentially disrupting mutations were found in multiple genes of this pathway in both patient tumors and cell lines. We also found that such mutations are associated with a poor outcome in HNSCC patients treated with chemoradiotherapy [16]. While DR provides cellular drug and radiation resistance through repair, it also governs genomic stability. Defects in DNA repair thus promote genomic instability, thereby enabling cancer cells to acquire new genetic traits, including those necessary to become therapy resistant. Indeed, the degree of a tumor’s genomic instability or DNA repair status is associated with poor prognosis in cancer and can therefore be of prognostic value [17,18]. In a previous study we showed that genomic instability measures vary between tumors of the oral cavity, larynx, and pharynx [15]. Measurements of genomic instability and correlates of DNA repair defects vary, but markers thereof can be obtained with genomic profiling. The total number of somatic point mutations (SPMs) in tumor DNA (“mutational burden”), alterations in ploidy, and/or the presence of chromosomal alterations—gains or losses—termed chromosomal instability (CIN) are possible markers of such factors [19].

Important questions, as to the prognostic value of the genomics in HNSCC, remain. Many studies focused solely on individual alterations. Even though frequent in HNSCC [4,5], the joint prognostic value of SPMs and CNAs in HNSCC has also not been assessed yet. In addition, previously studied cohorts often comprised tumors of various HNSCC sites. Patients were treated dissimilarly with a bias towards surgical resection of the tumor in most studies. However, HNSCC tumors from different anatomic sites have distinct genomic profiles [15]. Consequently, it remains to be determined which genetic markers are prognostic in specific sites treated with a uniform and contemporary modality. Importantly, since the large TCGA HNSCC study incorporated solely samples from resected HNSCC and included mostly oral cavity tumor specimens, oro- and hypopharyngeal carcinoma patients treated with standard-of-care definitive chemoradiotherapy are either not included or largely underrepresented in most current studies.

We therefore aimed to identify prognostic genetic factors of advanced stage pharyngeal squamous cell carcinoma treated with chemoradiotherapy. We performed a retrospective study based on pre-treatment tumor biopsies from 77 advanced stage HNSCC patients that all received cisplatin-based chemoradiation. Targeted DNA sequencing of these tumor biopsies was performed to identify gene mutations, and SPMs and CNAs were determined. These data were used to address the following questions: Are (1) mutated genes or co-occurring mutated genes, (2) mutational burden and/or (3) CIN markers associated with progression free survival (PFS)?

RESULTS

Patient Characteristics and Sequencing

In order to assess the potential prognostic value of frequent genetic factors, we genetically characterized 77 oro- and hypopharyngeal carcinoma samples (patient characteristics shown in Table 1). The median PFS in this cohort was 5.8 years (95% CI: 4.5–8.9). As reported previously in similar patient cohorts, PFS was significantly linked to tumor site and HPV status (Table 1, Supplementary Figure S1 and Supplementary Table S1). Tumor site and HPV status were therefore consistently included as independent variables in multivariable Cox models when testing for associations between genetic factors and PFS. Non-silent SPMs and CNAs were determined on targeted sequencing data of 556 genes (Supplementary Table S2) to identify gene mutations, deletions, and amplifications. In total, 416 non-silent SPMs were found in the sequenced genes, comprising mostly missense mutations (~80%, Supplementary Table S3).

In addition to individual gene mutations, we used the sum of all SPMs (including silent) in all sequenced genes in a tumor sample as a measure of its overall mutational burden. The median mutational burden was 5 per sample or 2.8 mutations per Mb (Supplementary Figure S2), a value comparable to previous findings by whole exome sequencing in HNSCC [20]. CNA analyses

on all targeted and sequenced genes provided ploidy estimates that served as a CIN marker. A total of 42% of the samples were classified as CIN+ according to this analysis. Together, our HNSCC data confirmed the mutational burden and chromosomal instability rich features that are characteristic to pharyngeal HNSCC [15].

"HNSCC Driver Genes"

First, we aimed to investigate the association of known "HNSCC driver genes", as determined by Iorio et al. and based on SPM and CNA data [4], with PFS (see Methods and Supplementary Tables S4,S5). For these PFS association analyses and consistent with the Iorio et al. identification criteria, only the genetic alteration type by which a gene was originally detected as a potential driver was considered [4]. For example, *CDKN2A* was identified as a potential HNSCC driver gene by its frequent presence of SPMs and deletions but not by amplifications. Amplifications were therefore not considered for *CDKN2A* sample classification, but SPMs and deletions were. The PFS association analyses were further restricted to frequently mutated genes only, a valuable requirement for any potential prognostic factor. In this study we therefore restricted the analyses to those genes which were mutated in the tumors of at least 10 patients, as this also allowed us to adequately fit a regression model [21,22]. Of all genes, the tumor suppressor gene *TP53*, the cell cycle regulators *CCND1* and *CDKN2A*, and the growth signaling gene *PIK3CA* met these criteria (Table 2). Notably, *CCND1* and *CDKN2A* alterations were only found in the HPV-negative tumor samples. We then used multivariable Cox models (with HPV and tumor site, see above) to investigate whether mutations in these genes were associated with PFS. Only in the *CCND1* analyses we found a trend towards shorter PFS for carriers of tumors with such amplifications (Table 2 and Supplementary Figure S3). This was the case regardless of whether HPV-positive samples were included or not (Table 2 and Figure 1A,B).

We next investigated pairs of co-occurring mutated genes. Tumors were classified as positive, in which both genes were mutated, and the rest as negative. Three pairs of co-occurring mutated genes were tested after applying the same restriction as above, of minimal 10 patients for each classification (Table S6). We found that patients with tumors harboring co-occurring *CCND1* mutations (amplifications) and *CDKN2A* mutations (SPMs and deletions) had shorter PFS than the rest (multivariable Cox: HR 2.24, 95% CI: 1.09–4.61, $p = 0.028$) (Supplementary Figure S3C).

In HPV-positive tumors, no mutations were detected in the cell cycle regulators *CCND1* or *CDKN2A*. This is consistent with the lack of a requirement for such mutations since the HPV viral protein E7 affects cell cycle regulation. Importantly, the prognostic significance of co-occurring *CCND1* and *CDKN2A* mutations remained even after excluding HPV-positive tumors (multivariable Cox: $p = 0.029$, Figure 1C).

Taken together, we tested recurrently mutated “HNSCC driver genes” for an association with PFS. Although none of the individual genes reached significance, the co-occurrence of *CCND1* and *CDKN2A* mutations was associated with shorter PFS in our patient dataset.

CCND1 and CDKN2A Expression and Patient Outcome

Of the 15 tumors that carried co-occurring *CCND1* and *CDKN2A* mutations, all carried a *CCND1* amplification, 1/15 had a *CDKN2A* frameshift SPM, and 14/15 a *CDKN2A* deletion. Gene copy number aberrations were thus the primary gene alteration in this HNSCC subgroup that displayed significantly shorter PFS. We reasoned that these CNAs might be reflected in RNA expression changes which could therefore be used as a prognostic biomarker. To test whether this was feasible, we analyzed RNA-seq data available for 74 of the 77 tumors. Indeed, we found that *CDKN2A* deletion was associated with low *CDKN2A* expression (p < 0.001) and *CCND1* amplification with high *CCND1* expression (p < 0.001) (Figures 2A–C). However, *CCND1* and *CDKN2A* expression could not accurately discriminate tumors with co-occurring *CCND1* amplifications and *CDKN2A* deletions (Figure 2C).

We then classified tumors into low and high expression groups using the median expression as the threshold value. High *CCND1* expression and *CDKN2A* expression showed an association with poor prognosis in univariate models based on all samples (log–rank p = 0.032 and p = 0.01, respectively). However, this was due to HPV-positive tumors and hence lost in the multivariable analyses (Figure 2D,E). Tested as a numeric (continuous) variable, as opposed to the median split, expression was not significantly associated with PFS either (data not shown). A co-occurrence analysis based on the median expression classifications did not reveal an association with PFS either (Figure 2F).

Taken together, the prognostic value of *CCND1* and *CDKN2A* was evident in the gene mutational analysis but not in the expression analyses. In fact, RNA expression analyses were unable to discriminate between *CDKN2A* wildtype and deleted *CDKN2A* in the HPV-negative samples (Figure 2B). At this stage, it is difficult to assess whether this may be a consequence of technical sensitivity issues due to stromal contamination.

Mutational Burden and CIN

We next aimed to investigate the prognostic value of genomic instability and DR defect related markers, as these are common in HNSCC [15]. Limited by the lack of whole exome or genome sequencing data, we used the estimates of mutational burden and CIN determined by ploidy estimates as described in the Materials and Methods Section. We find that in comparison to HPV-positive tumors, HPV-negative tumors had a higher mutational burden (p = 0.006) and were more often classified as CIN+ (p < 0.001) (Table 3).

We then tested whether mutational burden and CIN were associated with PFS using multivariable Cox models. When divided into "high" and "low mutational burden" groups according to the median mutational burden value, patients with a high tumor mutational burden had a trend towards shorter PFS (multivariable Cox with tumor site and HPV: HR 1.87, 95% CI: 0.91–3.86, $p = 0.089$) (Figure 3A and Table 3). CIN was associated with PFS as patients with CIN+ tumors had shorter PFS than those without (multivariable Cox: HR 2.3, 95% CI: 1.12–4.71, $p = 0.023$) (Figure 3B and Table 3).

Although CIN+ tumors had a "high mutational burden" classification more often than tumors without CIN (56% versus 29%, $p = 0.02$), this did not always co-occur. Importantly, when included into the same multivariable model, with tumor site and HPV status as co-variables, CIN remained significant, and mutational burden showed a trend ($p = 0.019$ and $p = 0.07$, respectively). In this model, both CIN+ (HR 2.35, 95% CI: 1.15–4.81) and high mutational burden (HR 1.91, 95% CI: 0.95–3.86) were associated with shorter PFS. Importantly, it was the patients with tumors classified by both genetic features, a CIN+ and a high mutational burden, who had the shortest PFS (multivariable Cox: HR 3.63, 95% CI: 1.41–9.31, $p = 0.007$) (Figure 3C).

Next, we combined all three prognostic genetic factors to assess whether they were independent predictors of PFS. We first tested for possible associations of co-occurring *CCND1* and *CDKN2A* mutations with mutational burden and CIN in the samples and found those alterations to be more prevalent in the HPV-negative samples (Supplementary Figure S4). Given the association of these genetic alterations with each other and with HPV status (Supplementary Figure S4), it is worthwhile to assess their independent prognostic value in a multivariable analysis. In fact, the association of high mutational burden and CIN with short PFS remained as a trend in multivariable Cox models that combined mutational burden, CIN, co-occurring *CCND1* and *CDKN2A* mutations, tumor site, and HPV status (with $p = 0.079$ and $p = 0.059$, respectively).

Taken together, we found that the presence of CIN was associated with short PFS in our cohort (in an HPV-status independent manner). Furthermore, the combined presence of CIN and a high mutational burden marked patients with a particular poor prognosis.

DISCUSSION

We found that a high mutational burden showed a trend towards poor prognosis and that CIN/aneuploidy was associated with short PFS in our cohort of advanced stage oro- and hypopharynx carcinoma patients treated with chemoradiotherapy. A high mutational burden has been linked to poor prognosis in surgically resected HNSCC [8] and other cancer types [13,23]. Similarly, the association between CIN and poor prognosis has been demonstrated in various cancer types

[24–26], including surgically resected HNSCC [27]. Mutant-allele tumor heterogeneity (MATH), a mutation-based measure of genetic heterogeneity, was found to be associated with poor prognosis in resected HNSCC [28]. Our findings thus confirm this poor prognostic pattern also for HNSCC patients who received chemoradiotherapy. They suggest that mutational burden and CIN are potential biomarkers in advanced stage HNSCC treated with chemoradiotherapy. DNA repair defects can cause genomic instability [29,30] and can therefore correlate with measurements thereof, such as mutational burden or CIN. Our results are therefore consistent with our previous reports demonstrating the presence of DNA repair defects in HNSCC and its association with poor prognosis [16]. Although therapeutic exploitation of CIN is currently not feasible in the clinic, strategies to do so are under investigation [31,32]. Tumor DNA repair defects on the other hand can be exploited by the use of DNA repair inhibitors [33].

The mutation frequencies in the “HNSCC driver genes”, defined by Iorio et al., that were observed in our study are consistent with similar HNSCC reports [3,8,10,11,34]. However, not all “HNSCC driver genes” [3,4] were sequenced in this patient cohort. The prognostic value of the remaining genes still needs to be determined for patients treated with chemoradiotherapy in future research. Individually, none of the mutated genes that were analyzed was associated with PFS in our cohort. This was despite, or because of, stringent statistical requirements for analysis (minimum of 10 patients per group). An improved outcome of patients with *NOTCH1*-mutated tumors was reported in a similar cohort [11]. Due to the limitation in cohort size, this finding was based on two events (deaths) in 12 patients with *NOTCH1*-mutated tumors. *NOTCH1* was part of our target genes but did not meet the frequency criteria in our cohort. Yet, when testing this reported association in our cohort, we did not observe an association between *NOTCH1* mutations status and PFS (data not shown). It should be noted, however, that a low patient and/or event number impedes strong conclusions from low prevalence mutations outcome association analyses in the medium-sized cohorts that are available, including ours [21,22]. Yet, our cohort had similar clinical and genetic features as comparable cohorts. Indeed, to mitigate the uncertainty caused by the sample size, we only tested associations with those mutated genes that were present in at least 10 patients with a PFS event.

Furthermore, poor outcome of patients with *TP53*-mutated tumors has been reported in multiple studies [8,10,11], even after correcting for HPV-status. However, each of these studies selected a different subset of *TP53* SPMs, as identified by different algorithms aimed at discriminating harmful from benign *TP53* SPMs. This difference in the classification of *TP53* mutations hampers comparison between studies. One should also note that it remains uncertain whether HPV-negative tumors classified as *TP53*-wildtype are truly *TP53*-wildtype as multi-codon deletions are difficult to detect with short-read sequencing technologies.

The discrepancies between studies underscore the challenges of HNSCC prognostic biomarker research that lag behind those of other cancer types [13,35]. Owing to a high heterogeneity of the disease, it is difficult to obtain large cohorts of patients treated with the same modality and with homogenous tumors (in terms of stage and site). Comparing oral with pharyngeal HNSCC, our previous study [15] pointed to the presence of discrete genomic features in each site. Patient outcome parameters will be also largely affected by the different treatment options in a tumor biology and genomics dependent manner, and this highlights the need for treatment uniformity in such cohorts. We therefore opted for a homogenous cohort of patients to assess the impact of frequent HNSCC gene mutations.

The association of co-occurring *CCND1* and *CDKN2A* mutations with short PFS is remarkable, given the similar role in cellular biology. The protein product of *CCND1*, cyclin D1, forms a complex with cyclin-dependent kinases (CDKs) *CDK4* or *CDK6*. This complex promotes cell cycle progression through the G1/S checkpoint but can be inhibited by the protein product of *CDKN2A*, p16. As either *CCND1* amplification or *CDKN2A* loss could promote cell cycle progression, these mutations seem redundant at first sight [36]. The high prevalence of co-occurring *CCND1* and *CDKN2A* mutations in HNSCC does however suggest an additional growth advantage [36,37] and is supported by our findings and in vitro and in vivo data of others [38,39]. Supported by immunohistochemistry, FISH or RT–PCR, four earlier studies reported that the co-occurrence of *CCND1* overexpression or gain and the *CDKN2A* loss of expression, under-expression, or deletion marks a specific group in HNSCC with poor prognosis [40–43]. This is consistent with our findings based on gene mutation analyses. The deletion of *CDKN2A* may influence the interaction opportunity of survivin with CDK4 and further supports cell proliferation driven by the cyclin D amplification [44]. Survivin also suppresses apoptosis and its overexpession has been shown to be associated with poor prognosis in surgically resected HNSCC and OSCC [45,46].

Strong genetic biomarkers can guide clinical decision-making in the future. Patients with a confirmed poor prognosis may be eligible for a different, intensified, or targeted treatment and/or may benefit from increased surveillance. Genetic biomarkers can also provide clues for such alternative treatments and administration of targeted agents. *CCND1* overexpression due to amplification and its cellular activity or loss of *CDKN2A* is for example targetable with *CDK4/6* inhibitors [4,47,48]. One such inhibitor, palbociclib, is currently being tested for HNSCC [49].

CONCLUSIONS

Our study shows that mutational burden estimates, CIN/aneuploidy markers, and co-occurring *CCND1* and *CDKN2A* mutations in advanced stage oro- and hypopharyngeal HNSCC are associated with patient outcomes. This highlights their potential as prognostic factors in advanced stage HNSCC patients treated with chemoradiotherapy and may have implications for targeted therapies or immunotherapies.

MATERIALS AND METHODS

Patients

Pretreatment HNSCC samples were collected by biopsy and fresh frozen for biobanking; they were available for retrospective analyses. All patients gave their informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee, the Institutional Review Board of the Netherlands Cancer Institute (P05MD1). Samples were obtained from 77 patients with oro- and hypopharyngeal carcinoma and treated with cisplatin based chemoradiotherapy at our institute between 2001 and 2010. Biopsy material with at least 50% tumor cells, as determined on H&E (haematoxylin and eosin stained) sections, was processed for DNA extraction. Matched normal samples were unavailable for the majority of tumors, and genomic analyses were therefore performed on tumor samples only. Patient cohort and tumor characteristics are described in Table 1.

Sequencing and Bioinformatics Protocol

Details of the DNA and RNA sequencing and bioinformatics protocols are specified in the Supplementary Methods [50–59]. In short, we performed target capture DNA sequencing of 556 human genes (Supplementary Table S2). HPV gene baits, to capture HPV DNA in the samples, were included in order to determine HPV status. We removed DNA sequence variants that were in any of three public SNP databases [54–56] and classified the remaining non-silent variants as SPMs. Homozygous deletions and focal amplifications were detected using the R package PureCN [57]. We used PureCN's ploidy estimates as a chromosomal instability (CIN) marker. These estimates were rounded to the nearest integer and values not equaling 2 were classified as CIN+ (presence of CIN).

"HNSCC Driver Genes"

We used the "HNSCC driver genes" published by Iorio et al. that were identified by a large cohort of HNSCC. All HNSCC patients in this study underwent surgical resection and thus were not treated with definitive chemoradiotherapy [4]. A total of 168 "HNSCC driver genes" were identified by algorithms that select genes with more non-silent SPMs than expected by chance

given various background mutation rates and processes (Supplementary Table S4). An additional 25 genes were selected from 33 regions identified as "drivers" by Iorio et al., by an analysis that selects regions with recurrent CNAs (Supplementary Table S5). We selected all genes that reside within these regions and that were annotated in the Cancer Gene Census [60] (Supplementary Table S5). A total of 27 out of the 168 SPM affected genes and 11 out of the 25 genes with recurrent CNAs (Supplementary Tables S4, S5) were captured in our targeted sequencing panel (Supplementary Table S2). These amounted to 35 unique "HNSCC driver genes", as three genes (*CDKN2A*, *EGFR*, and *NOTCH1*) were identified by both SPMs and CNAs.

Statistical Methods

Categorical and numerical variables were compared between groups with Fisher's exact test and the Wilcoxon rank–sum test, respectively. Median follow-up time was derived from a reverse Kaplan–Meier analysis [61]. Except for in-figure log–rank test *p*-values, all reported survival analyses were obtained with multivariable Cox proportional hazards models that were used to test for associations between the selected genomic factors and PFS. Each model included the genomic factor, HPV status, and tumor site. HPV status and tumor site were included since they are strong prognostic factors in advanced HNSCC and were also prognostic in our cohort (Table 1 and Supplementary Table S1). All statistical analyses were performed in the R environment for statistical computing.

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SUPPLEMENTARY INFORMATION

The supplementary information referenced in this chapter is freely available online at Cancers (https://doi.org/10.3390/cancers11040445 - see Supplementary Materials, below Conclusions).

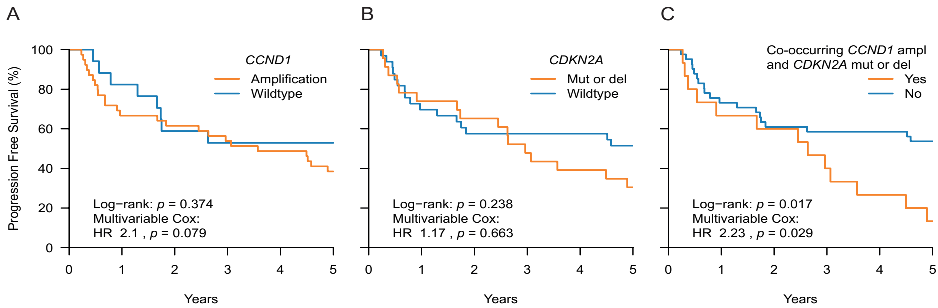

Figure 1. Progression free survival (PFS) according to genetic alterations in *CCND1* and *CDKN2A*.

Kaplan–Meier curves of advanced stage pharyngeal HNSCC patients classified according to tumor *CCND1* and *CDKN2A* status: (**A**) *CCND1* amplification versus wildtype. (**B**) *CDKN2A* mutation or deletion versus wildtype. (**C**) *CCND1* amplification co-occurring with *CDKN2A* mutation or deletion, versus wildtype for one or either of these two genes. In-figure legends of A–B state hazard ratios (HR) and corresponding *p*-values from multivariable Cox models that were fitted on all (n = 77) samples and included the tested genetic factor, HPV status, and tumor site. In-figure legend of C states the hazard ratio (HR) and corresponding *p*-value from a multivariable Cox model that was fitted on these HPV-negative samples only (n = 56) and included the tested genetic factor and tumor site. Note, all graphs display Kaplan–Meier curves of HPV-negative samples only (n = 56) with the corresponding log–rank test.

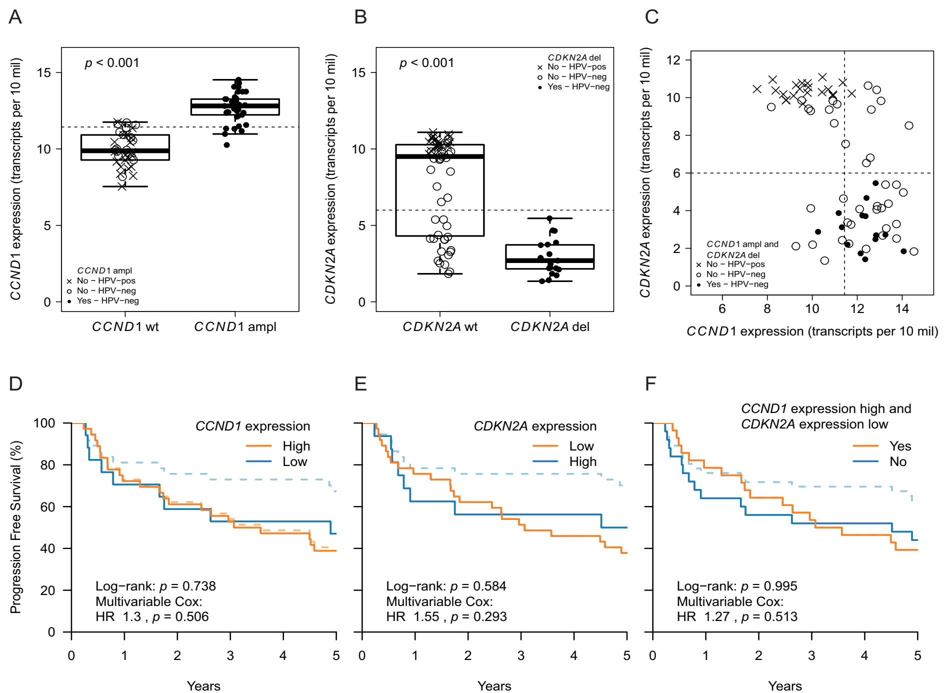

Figure 2. *CCND1* and *CDKN2A* expression and patient outcome.

(**A**) *CCND1* expression in tumors with ('ampl') and without ('wt') *CCND1* amplification. (**B**) *CDKN2A* expression in tumors with ('del) and without ('wt') *CDKN2A* deletion. (**C**) *CDKN2A* against *CCND1* expression per tumor sample, colored according to the presence (filled circles) or absence (open circles) of a co-occurring *CCND1* amplification and *CDKN2A* deletion. Dashed lines demarcate the median expression, according to which the samples received a high or low expression classification in D–F. (**D–F**) Kaplan–Meier curves of progression free survival (PFS) according to *CCND1* and *CDKN2A* expression status. Dashed lines represent all samples; solid lines represent HPV-negative samples only. Note, orange lines overlap in E–F. In-figure legends in D–F state hazard ratios (HR) and corresponding *p*-values from multivariable Cox models that were fitted on all ($n = 77$) samples and included the tested genetic factor, HPV status, and tumor site. In-figure stated log–rank tests correspond to the analyses in HPV-negative samples only.

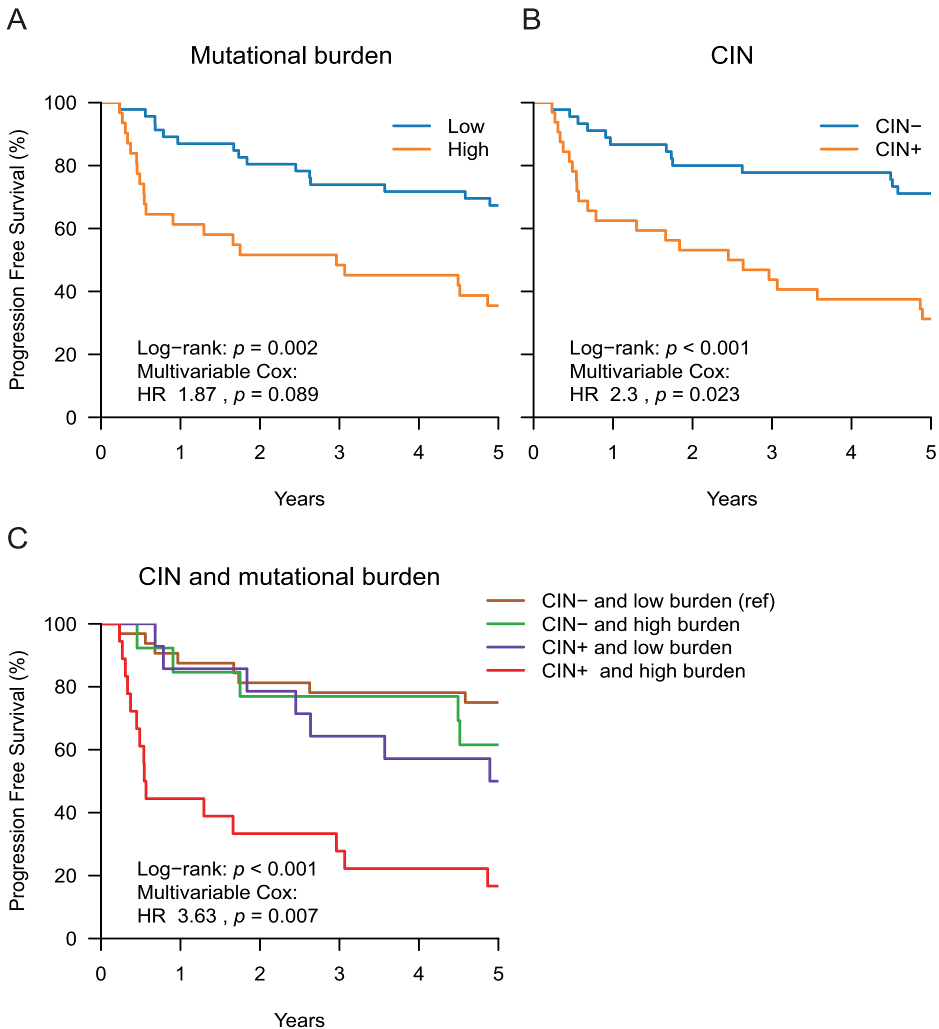

Figure 3. Progression Free Survival (PFS) according to mutational burden and chromosomal instability.

Kaplan–Meier curves of PFS after patient classification by the following genomic factors in their tumors: (**A**) High or low mutational burden, (**B**) presence (CIN+) or absence (CIN-) of chromosomal instability markers (CIN) and (**C**) mutational burden and CIN. In-figure legends state hazard ratios (HR) and *p*-values from multivariable Cox models that included the tested genomic factor, HPV status, and tumor site. The HR shown in (**C**) refers to patients that have tumors with a high mutational burden and that show presence of CIN in their tumors, compared to those with a low mutational burden and absence of CIN.

Table 1. Patient and tumor characteristics.

Follow-up and progression free survival (PFS) times in years.

Variable	All Patients	HPV-neg	HPV-pos	*p*-value
	N = 77	*N* = 56	N = 21	
	N (%)	*N* (%)	N (%)	
Gender				
Male	22 (29)	17 (30)	5 (24)	0.778
Female	55 (71)	39 (70)	16 (76)	
Tumor site				
Hypopharynx	28 (36)	28 (50)	0 (0)	< 0.001
Oropharynx	49 (64)	28 (50)	21 (100)	
Disease stage				
III	11 (14)	9 (16)	2 (10)	0.717
IV	66 (86)	47 (84)	19 (90)	
cT classification				
T1-T3	49 (64)	33 (59)	16 (76)	0.192
T4	28 (36)	23 (41)	5 (24)	
cN classification				
N0-N2a	24 (31)	20 (36)	4 (19)	0.181
N2b-N3	53 (69)	36 (64)	17 (81)	
Smoker				
Former	20 (26)	12 (21)	8 (38)	< 0.001
Never	7 (9)	1 (2)	6 (29)	
Unknown	2 (3)	2 (4)	0 (0)	
Yes	48 (62)	41 (73)	7 (33)	
Alcoholic consumption				
Former alcoholic	13 (17)	13 (23)	0 (0)	0.005
Never	10 (13)	4 (7)	6 (29)	
Unknown	2 (3)	2 (4)	0 (0)	
Yes	52 (68)	37 (66)	15 (71)	
Median age at diagnosis (range)	58 (27–78)	58.5 (37–78)	57 (27–77)	0.175
Median follow-up (95% CI)	8.2 (6.7–10.5)	9.3 (7.8–NA)	6.6 (5.9–10.2)	0.113
Number of PFS events (%)	47 (61)	43 (77)	4 (19)	< 0.001
Median PFS (95% CI)	5.8 (4.5–8.9)	4.5 (1.8–6.2)	NA	< 0.001

Table 2. Prevalence of selected driver gene mutations and association with progression free survival (PFS).

		Prevalence				Survival Analysis [b]	
		All Patients	HPV-negative	HPV-positive		PFS	
		N = 77	*N* = 56	*N* = 21			
Gene	Mutation [a]	*N* (%)	*N* (%)	*N* (%)	*p*-value	HR	*p*-value
TP53	SPM	48 (62)	47 (84)	1 (5)	< 0.001	0.87	0.753
CCND1	A	39 (51)	39 (70)	0 (0)	< 0.001	2.1	0.079
CDKN2A	SPM or D	23 (30)	23 (41)	0 (0)	< 0.001	1.17	0.663
PIK3CA	SPM	16 (21)	10 (18)	6 (29)	0.35	1.16	0.732

[(a)] SPM = Somatic Point Mutation, A = Amplification, D = Deletion. [(b)] Multivariable Cox model with the additional independent variables HPV status and tumor site.

Table 3. The association of mutational burden (total number of somatic point mutations) and presence of the chromosomal instability marker (CIN) with progression free survival (PFS).

	Prevalence				Survival Analysis [c]	
	All Patients	HPV-negative	HPV-positive		PFS	
	N = 77	*N* = 56	*N* = 21			
Variable	*N* (%)	*N* (%)	*N* (%)	*p*-value	HR	*p*-value
Mutational burden [a]						
Per tumor	5 (0–16)	5 (1–14)	2 (0–16)	0.006	1.08	0.197
Mutational burden groups [b]						
High	31 (40)	26 (46)	5 (24)	0.116	1.87	0.089
Low	46 (60)	30 (54)	16 (76)			
CIN [b]						
Positive	32 (42)	30 (54)	2 (10)	< 0.001	2.3	0.023
Negative	45 (58)	26 (46)	19 (90)			

[(a)] Reports the median, range, and Wilcoxon rank–sum test *p*-value. [(b)] Reports the absolute number of tumors, percentage, and Fisher's exact test *p*-value. [(c)] Multivariable Cox model with HPV status and tumor site as additional independent variables.

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CHAPTER 6

General discussion

This thesis deals with multiple aspects of genetic factors in head and neck squamous cell carcinoma (HNSCC): their prevalence in different tumor sites, their detection, and their prognostic relevance. The investigated genetic factors can roughly be divided in two categories: DNA repair defects and genes frequently mutated in HNSCC. DNA repair defects are the main theme throughout this thesis, with a smaller role for frequently mutated genes. First, frequently mutated genes will therefore be discussed briefly, followed by a more comprehensive discussion of DNA repair defects. The period during which we conducted our research coincides with an ongoing surge of closely related publications. This discussion therefore aims to also describe relevant findings of others and their relationship to our research, and to suggest realistic paths towards precision medicine for HNSCC.

FREQUENTLY MUTATED GENES

Identifying the driver genes in cancer is an important goal of large-scale sequencing projects such as The Cancer Genome Atlas (TCGA) [1]. Mapping driver genes might contribute to our knowledge of cancer and could point to novel therapeutic targets and biomarkers. Sophisticated computational methods have been developed to identify driver genes from DNA sequencing data. The general principle behind many of these methods is to select genes that are mutated significantly more frequently than expected by chance, after correcting for factors such as gene length and mutational background processes [2]. By now, large numbers of tumors from different cancer types have been sequenced and their data integrated and extensively analyzed. Therefore, it is likely that the vast majority of driver genes have been discovered by now [3]. Analyses on the pathway level or on intergenic factors may reveal novel associations. However, overall, research is expected to move on from discovering driver genes to converting them into therapeutic targets and using them as biomarkers.

Depending on the dataset, applied method, and level of evidence, the number of significantly mutated genes in HNSCC ranges between 10-200 [4–6]. Although these genes are more frequently mutated than expected by chance, most are mutated infrequently in absolute terms. For example, only 17/168 genes identified as significantly mutated by Iorio et al. in TCGA HNSCC samples are mutated in more than 5% of these samples [5]. Moreover, only a minority of all significantly mutated genes are established driver genes. The rest should be considered putative driver genes, awaiting functional validation for involvement in cancer [3,6,7].

Individually, infrequently mutated putative driver genes are not ideal candidates for practical purposes. Regarding targeted therapies, it might not be economically viable to develop drugs against such infrequently mutated genes. In addition, recruiting sufficient eligible patients in clinical trials to demonstrate efficacy might prove difficult; although novel trial designs have been

developed to address this issue [8,9]. Furthermore, infrequently mutated putative driver genes are not particularly promising biomarker candidates. Firstly, in data-driven research there is a low *a priori* probability that a mutated gene has a strong prognostic effect, especially when it is not therapeutically targeted. Secondly, weaker prognostic factors require more cases with this factor to reach significance (Figure 1) [10]. For example, whereas a mutated gene with a hazard ratio of 2 requires ~20 patients with this factor to reach significance, a hazard ratio of 1.2 requires ~200 patients. For infrequently mutated genes such numbers are hard to come by, especially in a disease with relatively low incidence such as HNSCC. Solutions to these issues are to integrate and analyze infrequently mutated genes at the pathway level or to use a hypothesis-driven approach. We used a combination of these solutions in Chapters 2 and 3. Various data led us to hypothesize that the homologous recombination (HR) and Fanconi anemia (FA) pathways might be involved in HNSCC. Although most HR/FA genes are mutated infrequently in HNSCC [4], this amounts to a substantial proportion of mutated tumors at the pathway level. Consequently, we were able to detect an association of HR/FA pathway DNA variants with a functional DNA repair readout and patient survival.

Most of the significantly mutated genes that are mutated in a substantial proportion of HNSCC are established driver genes (Table 1). As discussed in Chapter 1, established driver oncogenes are candidates for targeted therapy. There are three oncogenes among the frequently mutated driver genes in HNSCC: *CCND1*, *EGFR*, and *PIK3CA* [6]. Targeted therapy against EGFR is already routinely used in HNSCC treatment in the form of cetuximab. In contrast, targeted therapies against the protein products of *CCND1* and *PIK3CA* are still under investigation. Once these targeted therapies have been developed, *CCND1* and *PIK3CA* mutations might become predictive biomarkers. For example, it seems intuitive that *PIK3CA* activating mutations would be associated with PI3K inhibitor efficacy. However, in practice such associations are not always found. This is exemplified by the lack of association of *EGFR* amplification or overexpression with cetuximab response in HNSCC [11,12]. When oncogene mutations are not predictive of response to the associated targeted therapies, further investigation of biological resistance and response mechanisms is required.

We found that *CCND1* amplification is more common in laryngeal and pharyngeal HNSCC than in oral HNSCC (Chapter 4). Consequently, targeted *CCND1* therapies such as CDK4/6 inhibitors might show more efficacy in patients with laryngeal and pharyngeal HNSCC. Clinical trials of CDK4/6 inhibitors might strive to primarily recruit patients with laryngeal and pharyngeal HNSCC accordingly. Furthermore, we found that co-occurring *CCND1* amplification and *CDKN2A* inactivating (copy number and somatic point) mutations were associated with poor prognosis in a cohort of HNSCC patients treated with chemo-radiotherapy (Chapter 5). Interestingly, it has been noted that *CDKN2A* loss might predict sensitivity to CDK4/6 inhibitors [3,5]. Therefore, co-

occurring *CCND1* and *CDKN2A* mutations might thus become a prognostic or predictive biomarker in the future.

Finally, it cannot go unnoticed that the majority of frequently mutated driver genes in HNSCC are tumor suppressor genes. This means that the majority of mutated driver genes in HNSCC is not directly targetable with molecular inhibitors. However, loss of a tumor suppressor gene might be exploited via the synthetic lethality principle [13,14]. HR deficiency (HRD) is a prime example of an alteration that can be targeted with synthetic lethality, and substantial part of this thesis pertains to HRD in HNSCC.

HOMOLOGOUS RECOMBINATION DEFICIENCY

Pathogenic heterozygous germline mutations in *BRCA1* and *BRCA2* (*BRCA1/2*) predispose female carriers to breast and ovarian cancer [15]. During tumorigenesis the wild-type allele is lost [16,17], and together with it the gene's various cellular functions. Consequently, these familial-BRCA cancers have characteristic phenotypes; notably genomic instability and a sensitivity to platinum-based drugs [16]. The organ-specific cancer predisposition conferred by *BRCA1/2* mutations led scientists to question whether these phenotypes are also present in sporadic cancers of these organs. This concept has become known as BRCAness, originally defined as 'the phenotypes that some sporadic tumors share with familial-BRCA cancers' [16]. Since then, BRCAness has been confirmed not only in breast and ovarian cancer, but in other cancers as well [17].

Tumor HRD is thought to promote the BRCAness phenotype. BRCA1 and BRCA2 are important for HR-mediated DNA repair, and loss of either results in HRD. HR is involved in the faithful repair of lesions that cause stalled or collapsed replication forks and double-strand breaks. Cells unable to repair these lesions with HR therefore need to rely on less accurate repair mechanisms, that result in the accumulation of DNA damage and/or cell death [18]. Consequently, HRD causes high levels of genomic instability, which can contribute to the acquisition of driver mutations, but can also be exploited by drugs that induce lesions that require HR for repair, or by drugs that target pathways that are synthetic lethal with HRD. Two notable classes of such drugs are platinum-based drugs and poly(ADP-ribose) polymerase (PARP) inhibitors. Platinum-based drugs form interstrand crosslinks (ICLs) of the DNA. ICLs cause stalled replication forks and therefore require HR for repair. Cells with HRD are thus unable to accurately repair these ICLs and subsequently undergo cell death via various paths (Figure 2) [18]. PARP inhibitors have a different mechanism of action (Figure 3) [19]. The enzyme PARP1 binds at the site of single-strand breaks of the DNA and initiates DNA repair. PARP inhibitors prevent the release of PARP1 ("trapping") and replication forks stall when they encounter this complex. Both platinum-based drugs and PARP

inhibitors thus work by causing replication forks to stall and cause a characteristic sensitivity in tumor cells with HRD.

The HR and FA pathway work in close conjunction to remove ICLs. FA is a genetic disorder caused by bi-allelic germline mutations in FA genes and is characterized by developmental abnormalities, bone marrow failure, sensitivity to crosslinks, and a predisposition to cancer, notably HNSCC. A gene is classified into the FA family (by assigning a name to the gene that starts with the characters 'FA') when its bi-allelic germline mutation is found in at least two FA patients [20]. Due to this requirement for FA nomenclature, new FA genes continue to be identified and many HR genes have now also received a FA name. For example, *BRCA1*, *BRCA2*, and *RAD51* are also known as *FANCS*, *FANCD1*, and *FANCR*, respectively. Importantly, loss of any of the FA genes results in genomic instability and sensitivity to ICLs, similar to HR genes. FA and HR are thus closely related by cellular function and nomenclature. Their distinction is even less clear in the context of BRCAness, as genomic instability and sensitivity to crosslinking agents are centrally important BRCAness features. Consequently, many genes that modulate the response to crosslinking agents, including all FA genes, have been incorporated in the list of BRCAness genes [17]. In this discussion we will consistently denote this set of genes with the abbreviation 'HR/FA' to ensure clarity. Even though HRD, as often described in the context of BRCAness, does not necessarily stem from a defect in an HR gene, we will consistently write HRD, as this has become the common terminology in the literature.

Remaining questions

Two observations prompted our group to investigate the presence of HRD in HNSCC. First, FA is associated with a 500 fold increased risk of HNSCC [21], leading to a cumulative incidence of 40% by 40 years [18]. This raises the question whether somatic inactivation of FA genes is present in sporadic HNSCC, akin to somatic inactivation of BRCA1 and BRCA2 in sporadic breast and ovarian cancer. Second, the addition of cisplatin to radiotherapy is associated with an approximately 8% decrease in cancer related death of HNSCC patients [22]. This benefit suggests that a subgroup of patients is particularly sensitive to cisplatin, possibly due to HRD.

In search of HRD in HNSCC, we first subjected 29 patient-derived HNSCC cell lines to multiple functional tests of DNA repair (Chapter 2). We found that a considerable proportion was DNA crosslink repair defective. We then sequenced the DNA of HR/FA genes from these cell lines in order to find DNA variants that were associated with, and possibly even causing, the observed DNA repair defects. We found that HR/FA gene variants with high variant allele frequency and a minor allele frequency below 2.5% were associated with mitomycin C sensitivity in vitro (Chapters 2 and 3). An identical variant selection procedure in patient tumor samples revealed an association of carriers with worse overall survival, but a favorable response to a

high cumulative cisplatin dose (Chapter 2). Furthermore, we extracted copy number-based HRD signatures from TCGA HNSCC tumor samples and found that HRD was present in a substantial portion. Furthermore, HRD was more common in laryngeal and pharyngeal tumor samples than in those of the oral cavity (Chapter 4).

We started our study of HRD in HNSCC in the mid 2000s. Since then, HRD has been intensively studied, particularly in breast and ovarian cancer. The insights from HRD studies can be used to shed light on important questions that remain after our research. These questions include: are bi-allelic alterations in HR/FA genes appropriate markers of HRD in HNSCC? What other type of omics-based HRD markers exist and what are their advantages and disadvantages (for use in HNSCC)? Using these markers, what is the estimated prevalence of HRD in HNSCC?

HRD MARKERS

How to best measure or characterize HRD is an unanswered question that continues to receive much attention [23]. This poses a theoretical problem, because if it is unknown what exactly needs to be measured, the validity of a measurement or marker cannot be assessed. Indeed, it is not uncommon to validate a novel HRD marker by correlating it with an existing marker. However, there is a practical purpose for HRD markers: to select patients who will likely respond favorably to platinum-based drugs and PARP inhibitors. In practice, HRD markers are therefore often developed and tested for their ability to predict response to these drugs.

HRD markers typically fall into two categories: those aiming to detect the genomic cause of HRD and those measuring a cellular phenotype caused by or associated with HRD. As loss of the wild-type allele causes HRD in pathogenic germline *BRCA1/2* mutation carriers, bi-allelic alterations in HR/FA genes are intuitive HRD markers in sporadic cancer as well. Although bi-allelic alterations encompass many different alteration combinations, the most common combination would be a mono-allelic point mutation (germline or somatic), followed by loss of the wild-type allele through a somatic loss of heterozygosity (LOH) event [24].

An alternative is to focus on a cellular phenotype associated with HRD. HRD is thought to leave characteristic imprints across the genome and to alter the transcriptome. Consequently, genomic and transcriptomic HRD markers have been developed. Here we will discuss and compare the following HRD markers: bi-allelic alterations in HR/FA genes, copy number signatures, gene expression signatures and mutational signatures.

HRD Markers: Bi-allelic alterations in HR/FA genes

Germline *BRCA1/2* mutation carriers typically lose the wild-type allele through LOH. The resulting genotype is known under various terms, including locus-specific LOH of BRCA and homozygous or bi-allelic mutation, alteration, or inactivation. In this context, alterations typically signify a variety of mutations that impair gene function, including nonsense, frameshift, and missense somatic point mutations, homozygous deletions, and epigenetic silencing. Bi-allelic HR/FA gene alterations are also found in tumors of patients that do not carry germline HR/FA mutations, resulting entirely from somatic events.

Given the requirement of loss of tumor suppressor gene function for carcinogenesis, bi-allelic alterations are intuitive HRD markers. A variety of studies underscore the validity of this approach. Experimental studies have shown that cell lines with bi-allelic *BRCA1/2* alterations are more sensitive to cisplatin [25] and PARP inhibitors [26–28] than those with mono-allelic or no mutations. Mutter et al. developed a functional *ex vivo* assay to identify primary sporadic breast tumors with HRD [29]. Integration with whole-genome sequencing data revealed that bi-allelic HR/FA alterations occurred in 8/9 HRD tumors. Recently, three studies investigated the association of HR/FA alterations with mutational and copy number-based HRD signatures [30–32]. Using TCGA breast cancer [30], pan-cancer [31], and breast and ovarian cancer [32] datasets, each found that tumors with bi-allelic alterations had higher signature scores than tumors with mono-allelic or no alterations. Furthermore, two studies tested bi-allelic HR/FA gene alterations as biomarkers of platinum-based drug response in the ovarian cancer dataset. Using different methods to identify bi-allelic alterations, both studies found that patients with tumors harboring bi-allelic alterations survived longer than the other patients; indicating favorable platinum response [31,32].

Clinical trials of platinum-based drugs and PARP inhibitors have begun to stratify patients according to mutations in *BRCA1/2* and other HR/FA genes [33–40]. These studies have found that such mutations were associated with a favorable treatment response, supporting their value as predictive biomarkers. However, the majority of these studies did not stratify or even discriminate between mono- and bi-allelic alterations. Generally, deletions were required to be bi-allelic, but this requirement for bi-allelic inactivation was not extended to somatic point mutations. Given the absence of discrimination between mono- and bi-allelic alterations, these clinical trials might seem to contradict that mainly bi-allelic alterations cause HRD. However, except one, these were all ovarian and breast cancer trials, and the majority of HR/FA gene mutations in ovarian and breast cancer are coupled with LOH - thus bi-allelic alterations [32,35]. Therefore, tumors with bi-allelic HR/FA alterations may have driven the association with favorable response to platinum-based drugs and PARP inhibitors.

There are various explanations why clinical trials have not yet discriminated between mono- and bi-allelic alterations. Methods that enable such discrimination are novel and were unavailable when most of these trials were conducted. In addition, such methods often require whole-exome sequencing data, which may be too expensive in large clinical trials. Furthermore, these methods can give contradictory predictions and are by some considered too immature for use in a clinical trial or setting [41].

In short, research of (mainly) breast and ovarian cancer has reported findings that echo our results in HNSCC. Similar to Mutter et al., we found that bi- but not mono-allelic HR/FA gene variants were associated with functional HRD in HNSCC cell lines (Chapters 2 and 3). We also found evidence that bi-allelic HR/FA gene alterations are associated with a favorable clinical response to cisplatin (Chapter 2), akin to findings in ovarian cancer [32,35]. Furthermore, we found that HR/FA gene mutations were associated with high HRD signature scores in the TCGA HNSCC dataset (Chapter 4), as was found in breast, ovarian, and pan-cancer datasets by others [30–32].

In general, bi-allelic HR/FA gene alterations can thus be used as HRD markers. This approach has its advantages and disadvantages. Its advantages (see above) include its intuitive link to the biological mechanism, proven value for breast and ovarian cancer, and low cost when only somatic point mutations are considered. Its disadvantages include variants of unknown significance (VUS), secondary mutations that cause drug resistance, and high cost when assessing multiple alteration types. In breast and ovarian cancer, the pathogenicity of many specific *BRCA1/*2 variants is known due to their association with hereditary cancer. Consequently, a *BRCA1/2* assay is already commercially available for breast and ovarian cancer (BRACAnalysis, Myriad Genetics). In contrast, most HR/FA gene variants are VUS in the context of HNSCC (Chapters 2 and 3). In vitro experiments could be used to elucidate the significance of these VUS, although these are costly and labor-intensive. Therefore, it has been proposed to use HRD signatures to reclassify VUS [31,42]. However, for biomarker purposes one could then forgo VUS reclassification altogether and simply rely on the HRD signature. Secondary mutations that restore HR function or otherwise cause anti-HRD-drug resistance are difficult to assess and therefore form another limitation of bi-allelic alterations as HRD markers. Finally, in order to avoid false negative classifications, it might be necessary to assess multiple types of alterations (e.g., methylation, translocations) instead of somatic point mutations only. Such an analysis is not only costly and labor-intensive, but will also increase the number of VUS.

In the long term, when HR/FA gene VUS have been resolved in HNSCC or variant effect prediction algorithms perfected, bi-allelic HR/FA gene alterations will likely be excellent HRD markers. In the short term however, it is appealing to detect HRD phenotypes that are independent of their

genomic cause, and use these as HRD markers. Copy number, gene expression, and mutational signatures have been developed for this purpose.

HRD Markers: Copy number signatures

HRD causes genomic instability, resulting in characteristic imprints across the genome. The central idea behind copy number-based HRD signatures is to capture characteristic imprints left in copy number data in order to detect HRD. Various copy number HRD signatures have been developed based on a variety of platforms, including BAC array, aCGH, SNP array, and NGS [43].

So far, HRD-score is the only HRD copy number signature that has been developed into a commercially available assay [44]. HRD-score combines three independently developed HRD measures, originally derived from breast and ovarian cancer SNP array data: LOH [45], telomeric allelic imbalance [46], and large-scale state transitions [47]. HRD-score has been shown to predict response to platinum-based drugs in breast cancer [44,48] but not to the PARP inhibitor Niraparib in ovarian cancer [49]. However, in the latter study patients derived benefit from the PARP inhibitor regardless of any HRD biomarker. Currently, HRD-score is marketed as a predictive biomarker of platinum-based drugs and PARP inhibitors.

We found that each of the three HRD-score components was increased in HNSCC subsites that respond relatively favorably to platinum-based chemo-radiotherapy (Chapter 4). Although HRD-score has not yet been validated or approved for HNSCC, we found that HRD-score correlates well with other markers of HRD in HNSCC (Chapter 4). Furthermore, Marquard et al. demonstrated that HRD-scores are high in HNSCC from a pan-cancer perspective, together with other cancer types that are commonly treated with platinum-based drugs [50].

Taken together, HRD-score is a relatively mature HRD biomarker, likely to be of value to HNSCC. Therefore, in vitro and clinical studies are warranted to validate HRD-score for use in HNSCC. Alternatively, an HNSCC specific copy number signature may be developed. MacIntyre et al. recently demonstrated that the copy number aberrations found in whole-genome shallow sequencing data of ovarian cancer can be deconvoluted into distinct signatures [43]. One of these signatures correlated strongly with *BRCA1/2* mutations. To identify an HNSCC-specific HRD signature, this approach could be applied to our HNSCC cell line panel and later validated in patient cohorts.

It has been noted that copy number signatures report the historical presence of HRD in a tumor, but not necessarily its current HRD status. As the transcriptome is more dynamic than the genome, HRD gene expression signatures are promising alternative HRD markers.

HRD Markers: Gene expression signatures

HRD cells need to rely on compensatory mechanisms to repair endogenous DNA damage, causing distinctive gene expression patterns. HRD gene expression signatures aim to capture such patterns in order to predict HRD in tumors. Since the commercialization of micro arrays various HRD gene expression signatures have been developed [51–54]. For example, Pitroda et al. recently developed an HR gene expression signature on 279 carcinoma cell lines [55]. Subsequently, this signature was validated in various breast and lung cancer cohorts. In these analyses, tumors with predicted HRD displayed more aggressive baseline features, but better response to DNA targeted therapies than the other tumors [55,56]. Recently, our group has developed an HNSCC-specific HRD signature (Essers et al., manuscript submitted).

There are several advantages and disadvantages to HRD gene expression signatures. A clear advantage is the relatively low cost of RNA sequencing. As the transcriptome is more dynamic than the genome, a supposed advantage of gene expression over genomic signatures is a more 'up-to-date' HRD readout, reflecting the current HRD status of the tumor. This enables these signatures to correctly classify tumors that were originally HRD, but regained HR proficiency through secondary mutations. However, given that HRD contributes to carcinogenesis, it is unclear why (a relevant proportion of) treatment naïve HRD tumors would regain HR proficiency. Furthermore, the opportunity for gene expression signatures to detect treatment-induced resistance is rather limited, given that routine tumor biopsies are mostly not feasible.

Disadvantages of gene expression signatures, in general, include inconsistency of identified gene sets and captured biological processes by different groups and lack of biological interpretability [57–60]. Furthermore, there are uncertainties regarding HRD gene expression signatures specifically. For example, it remains unknown what proportion of tumors with clinically relevant HRD would show characteristic gene expression pattern in response to endogenous DNA damage. Furthermore, loss of each HR/FA gene can cause HRD, but it is unknown whether loss of different genes produces distinct gene expression patterns. This last question has, however, been addressed in the context of mutational signatures.

HRD Markers: Mutational signatures

Processes that damage DNA (e.g. mutagen exposure) or impair repair thereof (e.g. HRD) largely underpin the abundance of mutations in cancer genomes. Mutational signatures aim to capture patterns of (small) mutations that characterize specific mutational processes. Thus, they essentially turn a genome into a biomarker for mutational processes. In principle, mutational signatures can be derived from any class of mutation, e.g., base substitutions, small insertions and deletions (indels) or genomic rearrangements. In practice, base substitution signatures

are used most frequently because these are the most abundant class of mutations in exome sequencing data.

In order to identify such base substitution signatures, Alexandrov et al. performed unsupervised analyses on pan-cancer exome sequencing data [61]. One of the identified signatures was frequently present in breast, ovarian, and pancreatic cancer; cancer types that frequently arise in germline *BRCA1/2* mutation carriers. Furthermore, this signature was associated with *BRCA1/2* mutations in breast and pancreatic cancer. This signature is therefore considered an HRD signature, commonly referred to as (COSMIC) Signature 3. Nik-Zainal et al. then extended this concept to indel and rearrangement signatures by analyzing whole-genome sequencing data from breast cancers [62]. In addition to confirming Signature 3, the authors identified two rearrangement signatures (RS3 and RS5) associated with tumors harboring bi-allelic *BRCA1/2* alterations. Interestingly, RS3 and RS5 could discriminate between tumors with *BRCA1* and *BRCA2* mutations.

Various findings have provided further evidence for the validity and usefulness of HRD mutational signatures. E.g., Polak et al., showed that Signature 3 was associated with bi-allelic, but not mono-allelic inactivation of *BRCA1/2* [30]. In addition, Signature 3 was found to discriminate between tumors with benign and pathogenic missense *BRCA1/*2 variants, indicating potential to aid HRD classification of tumors harboring VUS. Importantly, HRD mutational signatures are also present in tumors without *BRCA1/2* mutations, marking BRCAness [30,61,62]. Finally, a classifier combining multiple HRD mutational signatures (HRDetect) identified breast, ovarian and pancreatic tumors harboring *BRCA1/2* mutations with higher accuracy than the individual signatures [42].

Thus, mutational signatures are promising candidate HRD biomarkers. However, critics have pointed to a lack of causality in the development of these signatures. Mutational signatures are mathematical models fit to data from primary tumors, which are noisy model systems due to their unstable genomes and presence of multiple, simultaneously active mutational processes and subclones. Furthermore, validation of mutational signatures is often circular: signatures are considered HRD markers when associated with *BRCA1/2* mutations, which are HRD markers themselves. Thus, the evidence for HRD mutational signatures heavily hinges on associations found in primary tumor data. In vitro studies have therefore been conducted to establish a causal link between HRD and mutational signatures.

To this end, Zamborszky et al. performed whole-genome sequencing of $BRCA1^{+/+}$, $BRCA1^{+/-}$, $BRCA1^{-/-}$, $BRCA2^{+/+}$, $BRCA2^{+/-}$, and $BRCA2^{-/-}$ (chicken) cells [63]. $BRCA1^{-/-}$ and $BRCA2^{-/-}$ cells had higher rates of base substitutions, indels and rearrangements than the rest. Furthermore, the

mean mutation patterns of the *BRCA1*$^{-/-}$ and *BRCA2*$^{-/-}$ cells correlated most strongly with Signature 3, whereas this did not apply to the other cells. Finally, the *BRCA2*$^{-/-}$ cells contained more deletions and rearrangements than the *BRCA1*$^{-/-}$ cells. Zou et al. used CRISPR-Cas9 gene-editing to knock out a variety of DNA repair genes in the HAP1 cell line, including the HR-related gene EXO1 and the FA gene FANCC [64]. Again, the effect of gene knockouts on whole-genome patterns of base substitutions, indels and rearrangements was investigated. EXO1 knockout caused a base substitution signature similar to Signature 3 and an indel signature mimicking that of BRCA-deficient tumors. FANCC knockout caused a modest increase of indels, a pronounced increase in rearrangements, and an indel and rearrangement signature closely resembling that of BRCA-deficient tumors. Thus, even though *EXO1* or *FANCC* are not typical HR genes, their knockout resulted in mutational signatures resembling those of BRCA-deficient tumors. This observation furthermore demonstrates that loss of HR/FA genes other than *BRCA1/2* can result in HRD and associated mutational signatures. Other in vitro studies have provided similar support for tumor-derived mutational signatures: those associated with aflatoxin exposure signature [65], loss of mismatch repair gene MLH1 and base excision repair gene NTHL1 [66]. Taken together, these studies show that 'whole genome profiles of experimentally generated gene knockouts bear uncanny resemblances to whole genome profiles of primarily repair-deficient tumours' [64].

Of the three phenotypic HRD signatures discussed here (copy number, gene expression, mutational), mutational signatures are the most recent invention, yet have the most experimental support. On the other hand, HRD mutational signatures have not yet been tested in clinical studies, in contrast to copy number and gene expression signatures. However, given the resemblance between mutational profiles of cells lines and primary tumors, successful clinical application is most likely a matter of refinement, e.g. finding a threshold for classifying tumors as HRD or not. Current algorithms assign weights to HRD and other mutational signatures per tumor. A complementary threshold to classify an HRD signature as 'significantly present' in a tumor is necessary for its clinical application [67].

The insights from in vitro studies suggest additional possibilities for refining HRD mutational signatures. Currently, the effect of gene knockout on mutational signatures has only been recorded for *BRCA1*, *BRCA2*, *EXO1*, and *FANCC* knockout. These experiments have shown that each individual HR/FA is knockout produces a slightly different mutational signature [63,64]. Therefore, knockout experiments of the other HR/FA genes could reveal currently unknown HRD signatures or, more likely, variations on the known signatures. Ideally these experiments would be performed on all HR/FA genes to create a catalogue of HRD mutational signatures. Incorporating all of these signatures in an HRD classifier would likely improve classification accuracy.

The extensive validation of mutational signatures has implications for the research presented in this thesis. We found that an HRD mutational signature and three copy number signatures were more prevalent in laryngeal and pharyngeal than oral cavity tumors (Chapter 4). We noted that these signatures were developed for breast and ovarian cancer, and had yet to be validated for HNSCC. The in vitro studies discussed here provide such validation indirectly, by showing that HR/FA gene knockout results in known HRD signatures in cell lines from different tissue origins. Furthermore, the approach of using Signature 3 to classify tumors with HR/FA gene VUS could be a useful addition to our own research [30]. We detected bi-allelic HR/FA gene alterations in only a few of the HRD HNSCC cell lines (Chapter 2). These variants were thus a suboptimal HRD marker in HNSCC. It would be interesting to investigate whether Signature 3 (or another HRD mutational signature) is present in a higher proportion of HNSCC cell lines with functional HRD. Such research would provide evidence for the usefulness of mutational signatures as HRD markers in HNSCC and address general questions about the tissue-specificity of these signatures.

HRD Markers: Prevalence in HNSCC

Knijnenburg et al. [68] have made data publicly available that allows for an estimate of HRD prevalence in HNSCC, that can be compared with our data (15/77 patients or 19%, Chapter 2). This study provides HRD-scores for the pan-cancer TCGA dataset, including 482 HNSCC samples, which can be used to classify TCGA HNSCC samples. Here the threshold of HRD-score ³ 42 is used to classify samples as HRD, following the example of Telli et al. on breast cancer samples [44]. Remarkably, all but one HRD samples are HPV negative (50/390 versus 1/90, $p < 0.001$). Focusing on the 390 HPV-negative samples, 9.3% of oral cavity samples were HRD (24/258) and 19.7 % of the laryngeal and pharyngeal samples (L/P-SCC) combined (26/132). Laryngeal and pharyngeal samples are combined here because the latter are underrepresented in TCGA HNSCC data. Using the HRD-scores, the estimate of HRD prevalence in L/P-SCC is very similar to that found in P-SCC in Chapter 2 (19%).

Another study has made publicly available data of bi-allelic pathogenic alterations in 102 HR/FA genes of 499 HNSCC samples [31]. The authors defined pathogenicity narrowly: as frameshift, nonsense, start/stop codon changes, and splice site mutations, coupled with LOH. Combining this data with the HRD classification above gives an idea of how often such pathogenic alterations underpin HRD in HNSCC. Only 6% (3/50) of HPV-negative samples with HRD-score ³ 42 had bi-allelic pathogenic HR/FA gene alterations (and 3.5% or 12/340 of samples with HRD-score < 42). This finding emphasizes that identifying the genomic cause of HRD in HNSCC is not straight forward.

HRD Markers: Concluding remarks

The research discussed here shows that much progress has been made towards the detection of HRD in cancer. However, most of this research pertains to breast and ovarian cancer, not HNSCC. This should be seen as an advantage to HNSCC research, as it can apply the lessons learned from breast and ovarian cancer.

Identifying the precise genomic alterations causing HRD in HNSCC will require tremendous effort. Pathogenic bi-allelic HR/FA gene alterations do not explain HRD in the majority of HNSCC with HRD (Section *6.3.5*). This may be because these pathogenicity criteria were developed for breast and ovarian cancer, not HNSCC. However, removing this (classical) pathogenicity criterion results in VUS (Chapters 2 and 3), which are still not present in all HRD cases. Therefore, multi-omics explorative research and experimental studies are required to elucidate these VUS and identify the remaining causes of HRD in HNSCC. As such research would necessarily be limited to a sample of the population, a complete catalogue of causative alterations in HNSCC will remain elusive in the foreseeable future. Classification based on a limited number of confirmed causative alterations would result in a high false negative rate. A focus on signatures that capture a phenotype caused by HRD is therefore more pragmatic for classification.

HRD signatures based on different omics data (e.g., copy number, gene expression, mutational) each have their advantages and disadvantages (*6.3.2-4*). It remains to be determined which is the most useful for a clinical applicable HRD signature. However, it is likely that the first HRD signature that will find widespread clinical application in the future, will achieve this by effective commercialization rather than technological superiority.

The current HRD signatures are quantitative and require a threshold for tumor classification. Studies that have effectively used HRD signatures to classify tumors employed different strategies to determine this threshold [44,56]. An important question is whether these thresholds require tissue-specific calibration. The answer to this question would be particularly relevant for basket trials evaluating drugs targeting HRD. For HNSCC-specific, the HNSCC cell line panels with functional HRD measurements of us and others [69,70] could be used as starting points for calibrating HRD signatures. Ideally, an open-access catalogue of HR/FA gene knockouts and their effect on HRD signatures in cell lines of different cancer types should be created. This catalogue could then act as a gold standard for HRD marker development and validation.

Clinical validation of HRD signatures could possibly be achieved earlier when used as prognostic rather than as predictive biomarkers. Prognostic biomarkers carry information about patient survival that is independent of the administered treatment [71]. The value of HRD signatures as prognostic biomarkers for HNSCC could therefore be assessed using patients that receive the

current standard-of-care, which is a large number of patients. Predictive biomarkers relate to the (statistical) interaction between treatment and biomarker. Thus, their evaluation requires treated and untreated patients that are biomarker-positive and -negative. This design is difficult to realize for standard-of-care treatments (e.g., cisplatin), as it is unethical to withhold these from patients. However, our strategy of using treatment dose as a surrogate for treated/untreated (Chapter 2) shows that alternative solutions exist. Determining the predictive value of HRD signatures for more experimental drugs (e.g., PARPi) will not be hindered by this, and will most likely be elucidated first.

In short, our and others' research clearly demonstrate the presence of HRD in HNSCC. Developing an HRD marker for clinical application is the first step towards therapeutic exploitation of this tumor characteristic and thereby improving the lives of HNSCC patients through a precision medicine approach.

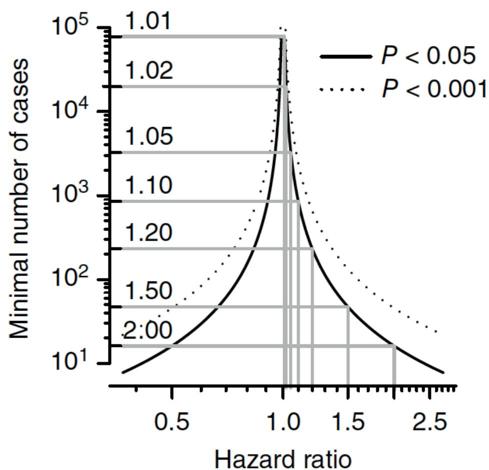

Figure 1. Power calculations of prognostic factors.

Graph relating the effect size (hazard ratio) of a prognostic variable to the absolute number of patients with the given factor that were required to reach significance in a random-effects model for overall survival (solid line, $p < 0.05$; dashed line, $p < 0.001$).
Figure and figure legend copied from Gerstung et al. [10].

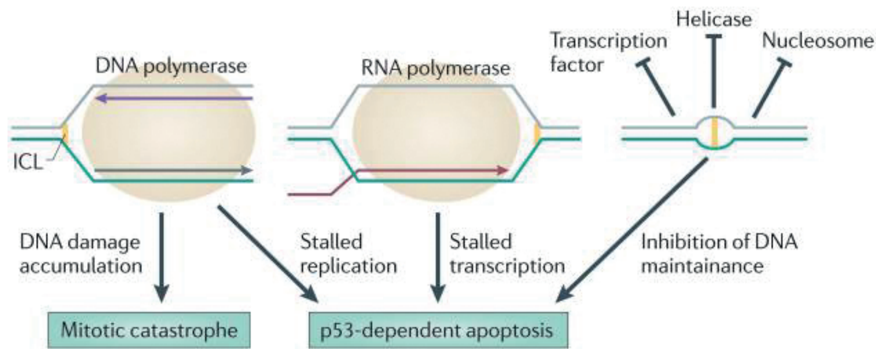

Figure 2. How ICLs kill tumour cells.

Interstrand crosslinks (ICLs) can block the progression of the replication fork by inhibiting the progression of the replisome. ICLs can stall transcription. ICLs may distort the structure of chromatin and prevent the access of DNA-interacting proteins. Tumour cell death can be induced by p53- and FAS ligand-dependent apoptosis (programmed cell death) or p53-independent mitotic catastrophe (death when apoptosis is absent but vital DNA integrity is lost).
Figure and figure legend copied from Deans and West [18].

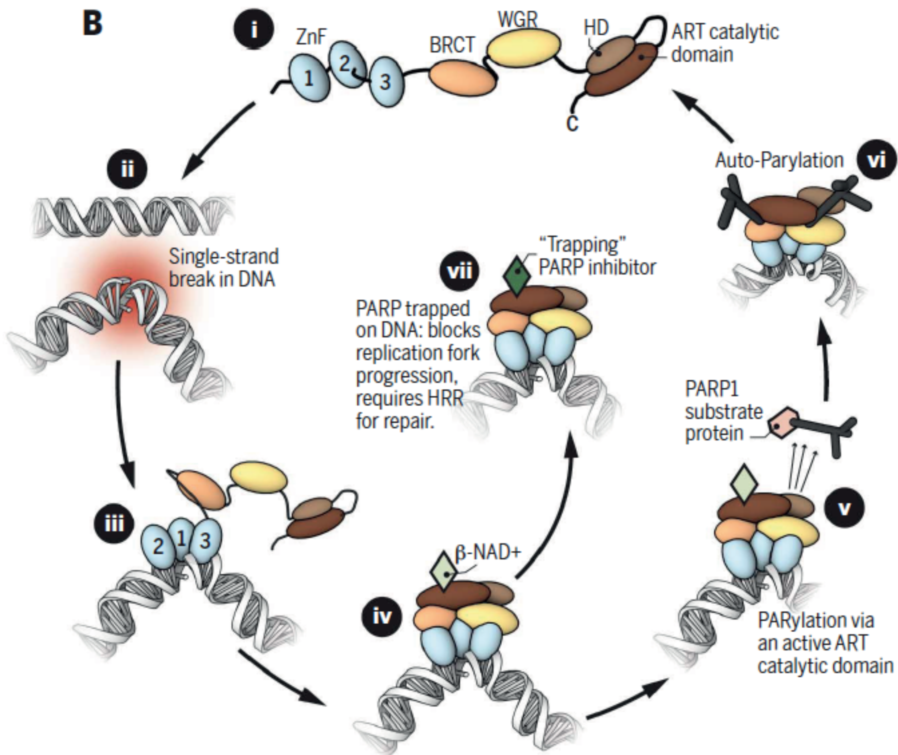

Figure 3. A model describing the PARP1 catalytic cycle.

(i) In its non-DNA bound state, PARP1 exists in a relatively disordered conformation, commonly referred to as "beads on a string". The domain structure of PARP1 is shown, including three zinc finger–related domains (ZnF 1, 2, and 3): the BRCA1 C-terminus domain (BRCT); the tryptophan-, glycine-, arginine-rich domain (WGR); and the catalytic domain, which encompasses two subdomains; a helical domain (HD) and an ADP-ribosyltransferase (ART) catalytic domain. In this non-DNA bound state, HD acts as an autoinhibitory domain preventing binding of the PARP-superfamily cofactor, b-NAD+, to its ART binding site. (ii) Damage of the DNA double helix often causes the formation of SSBs (predamaged and damaged DNA structures are shown); SSBs cause a change in the normal orientation of the double helix, which, in turn, (iii) provides a binding site for DNA binding PARP1 ZnF domains. The interaction of ZnF 1, 2, and 3 with DNA initiates a stepwise assembly of the remaining PARP1 protein domains onto the PARP1/DNA nucleoprotein structure, shown in (iv); this process leads to a change in HD conformation, and resultant loss of autoinhibitory function, thus allosterically activating PARP1 catalytic activity. (v) ART catalytic activity drives the PARylation of PARP1 substrate proteins (branched PAR chains are shown on a target protein), mediating the recruitment of DNA repair effectors, chromatin remodeling, and eventually DNA repair. (vi) PARP1 autoPARylation (likely in cis at SSBs but possibly in trans at other DNA lesions finally causes the release of PARP1 from DNA and the restoration of a catalytically inactive state [shown in (i)]. (vii) Several clinical PARPi, each of which binds the catalytic site, prevent the release of PARP1 from DNA, "trapping" PARP1 at the site of damage, potentially removing PARP1 from its normal catalytic cycle. These images are schematic; detailed structures and models of PARP1/DNA nucleoprotein complexes are described elsewhere.
Figure and figure legend copied from Lord and Ashworth [19].

Table 1. Genes with frequent and highly significant somatic genetic changes in HPV-negative HNSCC.

Table copied and modified from Leemans et al. [6].

Cellular process	Gene	Type of gene	Mutation frequency (%)	CNA frequency (%)
Cell cycle	*CDKN2A*	Tumour suppressor	22	32
	TP53	Tumour suppressor	72	1.4
	CCND1	Oncogene	0.6	25
Growth signals	*EGFR*	Oncogene	4	11
Survival	*PIK3CA*	Oncogene	18	21
	PTEN	Tumour suppressor	3	4
WNT signalling	*FAT1*	Tumour suppressor	23	8
	AJUBA	Tumour suppressor	7*	1
	NOTCH1	Tumour suppressor	18	4
Epigenetic regulation	*KMT2D*	Tumour suppressor	16	0.4
	NSD1	Tumour suppressor	12*	0.8

*Putative passenger mutation that requires further functional studies.

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Author contributions
Funding
Dankwoord

SUMMARY

Patients with advanced stage head and neck cancer squamous cell carcinoma (HNSCC) have a poor prognosis. Just about half of these patients are cured by the standard-of-care treatment with platinum-based chemo-radiotherapy, indicating an urgent need for improved treatment strategies. This thesis focuses on using genetic profiles of HNSCCs to guide and improve treatment selection.

Genetic alterations characterize cancer genomes and cancer treatments often work by targeting properties of cancer cells that are caused by their particular genetic alterations. Cancer genomics might therefore inform about the properties of a particular tumor and thereby the patient's prognosis, response to specific treatments, and treatment options.

A major part of this thesis is about particular tumor properties: DNA repair defects. These are interesting tumor properties from a clinical perspective, as cells with DNA repair defects have increased sensitivity to (platinum-based) chemotherapeutics and radiation. We specifically investigated defects of the homologous recombination (HR) and Fanconi Anemia (FA) pathways, as clinical data suggested such defects might be present in HNSCC.

We performed functional assays of DNA repair defects on HNSCC cell lines and found evidence of HR/FA defects in a considerable number of cell lines. We then performed DNA sequencing on these cell lines and on tumor biopsies of HNSCC patients that were treated with platinum-based chemo-radiotherapy. The presence of variants in HR/FA genes was associated with functional DNA repair defects in the cell lines and worse overall survival, but a favorable response to high cisplatin doses, in the patient cohort. In addition, we found that genomic scar signatures associated with HR/FA defects are strongly and frequently present in HNSCC. Moreover, these signatures were more often present in HNSCC subsites that have a relatively favorable response to definitive chemo-radiotherapy. We consider this work evidence for the presence of genetic FA/HR pathway defects in HNSCC and think that markers thereof could be of clinical value.

In addition to HR/FA defects in HNSCC, we also investigated more general aspects of the genetic profiles of HNSCC: frequently mutated genes and overall mutation rates. Oral squamous cell carcinomas patients typically have worse outcomes following definitive chemo-radiotherapy than laryngeal and pharyngeal squamous cell carcinomas (L/P-SCC) patients. This observation led us to question whether OSCC and L/P-SCC have a different tumor biology and hence different genetic profiles. Overall, the mutational profiles of OSCC and L/P-SCC showed many similarities. However, we identified genes that were more frequently mutated in either subsite. Moreover, we found that L/P-SCC have higher rates of genetic alterations than OSCC and scored higher on

genomic scar signatures associated with HR/FA defects. Finally, we investigated the prognostic value of frequently mutated genes and overall mutation rates among HNSCC patients treated with chemo-radiotherapy. We found that co-occurring *CCND1* and *CDKN2A* mutations, tumor mutational burden, and a marker of chromosomal instability were each associated with poor prognosis. From this research we conclude that the genetic profiles of HNSCC hold information that could be used to improve prognostication and individualize treatment protocols.

The thesis ends with a general discussion, describing closely related research that complementary to ours show how our findings might lead to precision medicine for HNSCC.

SAMENVATTING

Patiënten met een vergevorderd stadium plaveiselcelcarcinoom in het hoofd-hals gebied (HHPCC) hebben een slechte prognose. Aangezien slechts de helft van deze patiënten wordt genezen wanneer de standaardbehandeling wordt toegepast, bestaande uit een combinatie van platinumbevattende chemo- en radiotherapie, is er dringend behoefte aan nieuwe behandelstrategieën. Dit proefschrift richt zich op het gebruiken van genetische profielen van HHPCC om betere behandelbeslissingen te nemen.

Genetische veranderingen karakteriseren het kanker genoom en de behandeling van kanker grijpt vaak aan op eigenschappen die kankercellen hebben verkregen door deze genetische veranderingen. Het kanker genoom van een individuele patiënt bevat daarom mogelijk informatie over de eigenschappen van deze tumor en daarmee over de prognose van de patiënt, respons op specifieke behandelingen en behandelmogelijkheden.

Een groot deel van dit proefschrift gaat over specifieke tumor eigenschappen: DNA reparatie defecten. Dit zijn interessante eigenschappen vanuit een klinisch perspectief, omdat cellen met DNA reparatie defecten een verhoogde gevoeligheid hebben voor (platinumbevattende) chemotherapeutica en bestraling. In het bijzonder hebben we onderzoek gedaan naar defecten van de homologe recombinatie (HR) en Fanconi Anemie (FA) reparatie mechanismen, omdat klinische data suggereerden dat deze defecten mogelijk voorkomen bij HHPCC.

Functionele DNA reparatie experimenten op HHPCC cellijnen leverden bewijs voor de aanwezigheid van HR/FA defecten in een aanzienlijk aantal cellijnen. Vervolgens voerden we DNA sequencing uit op deze cellijnen en op tumorbiopten van HHPCC patiënten die werden behandeld met platinumbevattende chemo-radiotherapie. De aanwezigheid van DNA varianten in de HR/FA genen was geassocieerd met functionele DNA reparatie defecten in de cellijnen en met een slechtere overleving, maar een gunstige respons op hoge cisplatine doseringen, in het patiëntcohort. Daarnaast vonden we dat DNA copynumber profielen, die geassocieerd zijn met HR/FA defecten, sterk en frequent aanwezig zijn in HHPCC. Bovendien waren deze DNA copynumber profielen vaker aanwezig bij tumoren afkomstig uit hoofd-hals deelgebieden die relatief gunstig reageren op definitieve chemo-radiotherapie. We beschouwen dit werk als bewijs voor de aanwezigheid van genetische defecten van HR/FA mechanismen in HHPCC en geloven dat markers hiervoor klinische waarde kunnen hebben.

Naast HR/FA defecten in HHPCC hebben we ook onderzoek verricht naar meer algemene aspecten van de genetische profielen van HHPCC: frequent gemuteerde genen en het aantal mutaties per tumor. Patiënten met mondholtetumoren hebben vaak een slechtere uitkomst na

definitieve chemo-radiotherapie dan patiënten met larynx en farynx tumoren. Hierdoor vroegen wij ons af of mondholtetumoren een andere tumor biologie en genetische profielen hebben dan larynx en farynx tumoren. Globaal vertoonden de mutatie profielen van mondholtetumoren veel gelijkenis met die van larynx en farynx tumoren. We identificeerden echter specifieke genen die vaker waren gemuteerd in mondholtetumoren, dan wel in larynx en farynx tumoren. Bovendien vonden we dat larynx en farynx tumoren gemiddeld meer mutaties per tumor hadden dan mondholtetumoren en daarnaast hoger scoorden op DNA copynumber profielen, welke geassocieerd zijn met HR/FA defecten. Tot slot onderzochten we de prognostische waarde van frequent gemuteerde genen en het aantal mutaties per tumor voor HHPCC patiënten behandeld met chemo-radiotherapie. Tegelijk voorkomende mutaties in *CCND1* en *CDKN2A*, meer mutaties per tumor, en een marker van chromosomale instabiliteit waren allen geassocieerd met een slechtere prognose. Uit dit onderzoek concluderen we dat de genetische profielen van HHPCC informatie bevatten die gebruikt kunnen worden om nauwkeuriger prognoses te stellen en behandelplannen op maat te maken.

Het proefschrift sluit af met een algemene discussie, waarin nauw verwant onderzoek wordt beschreven dat complementair aan ons onderzoek laat zien hoe onze bevindingen kunnen leiden tot behandeling op maat voor HHPCC.

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Chapter 2

Fanconi anemia and homologous recombination gene variants are associated with functional DNA repair defects in vitro and poor outcome in patients with advanced head and neck squamous cell carcinoma

Study conceptualization and design:	CVens, MB, VW
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Chapter 4

Comparative genomic analysis of oral versus laryngeal and pharyngeal cancer

Study conceptualization and design:	CVens, MB
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Chapter 5

Genetic factors associated with a poor outcome in head and neck cancer patients receiving definitive chemoradiotherapy

Study conceptualization and design:	CVens, MB
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