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Review

Molecular analysis of surgical margins in head and neck cancer: More than a marginal issue

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SUMMARY

The relatively modest survival of patients surgically treated for advanced HNSCC can partly be explained by the development of local relapse. It is important that surgeons are able to predict which patients are at high risk to develop local relapse, since clinical management can be tailored. Local relapse after resection of a primary HNSCC is easily explained, when tumour is detected in the surgical margins and thus residual tumour is likely to remain in the patient, but the pathobiology is more complex in cases where the margins are histologically tumour-free. Molecular studies indicate that there are two different mechanisms responsible in these cases. First, small clusters of residual tumour cells that are undetectable on routine histopathological examination (known as minimal residual cancer: MRC) proliferate and this forms the basis of recurring cancer. A second cause of relapse is a remaining field of preneoplastic cells that is struck by additional genetic hits leading to invasive cancer. It is likely that within this field, that can be over 7 cm in diameter, the primary carcinoma has also emerged. Despite careful histopathological examination of the surgical margins of the primary carcinoma, it is at present not reliably possible to predict which patient will develop local relapse. Herein we focus on new developments regarding the analysis of margins, causes of local relapse, and how novel molecular techniques can be of help in a more accurate risk assessment. Critical analysis of the studies that have been published thus far shows that there is a list of promising markers, based on protein expression (immuno-histochemistry) and nucleic acid analysis. Further studies should be focused on validation and assessment of the clinical utility of these markers. Margin analysis should reveal whether one is dealing with residual cancer cells that might be treated by post-operative radiotherapy or with preneoplastic fields that remained behind. For this latter entity, there is no intervention available at present, except for a more intensive surveillance.

Introduction

The trends in incidence of head and neck squamous cell carcinoma (HNSCC) in the more developed countries appear to depend on the tumour-site; the incidence of cancer of the oropharynx and oral cavity is generally increasing, whereas that of laryngeal cancer seems to be decreasing.[1–4] Despite (chemo)radiotherapy being often the primary modality for advanced HNSCC, surgery is the primary mode of treatment for oral carcinoma. Surgery may be followed by (chemo)radiotherapy. A dilemma for the surgeon is to exactly delineate the area of excision. Local relapse has been reported to occur in up to 20% of cases.[5–7] This is one of the reasons that survival has only modestly increased in recent decades.[4] An important prognosticator is presence of carcinoma in or close to the surgical margins. This information will influence the decision whether additional treatment, usually post-operative (chemo) radiotherapy, is indicated as it is generally accepted that histologically tumour-positive margins signal high risk for tumour relapse. Important questions are how large the risk for relapse is when carcinoma is present close to the resection margin and when there is epithelial dysplasia in the mucosal margins. Moreover, relapses do also occur despite clear margins.

Herein we focus on the causes of local relapses of squamous cell carcinoma of surgically treated HNSCC patients and how investigation of margins with novel molecular techniques can be of help in more accurate risk assessment.

Local tumour relapse: two mechanisms

There are two tumour-biological explanations for the mechanism of local relapse.[8,9] First, there is the possibility that residual

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cancer remains behind, i.e., resection has been incomplete. In the majority of these cases routine histopathological investigation of the resection specimen will detect tumour tissue at the resection margin, making it likely that residual cancer is the cause of relapse, designated a local recurrence. It is, however, possible that the number of remaining tumour cells is too small to be detected by routine histopathology, a phenomenon also known as minimal residual cancer (MRC). A second possibility is that premalignant tissue has remained in the patient, and this eventually evolves into invasive cancer. The premalignant nature of the tissue can be suspected, when epithelial dysplasia is present as assessed with standard histopathology, and can be proven by molecular investigations. This latter type of research with genetic markers has revealed that there often is a clonal relationship between the primary tumour and the tumour-adjacent premalignant epithelium.[10] This information together with the results of molecular studies on leukoplakia[11,12] have led to the concept of the field-carcinogenesis model.[13] The basic principle of this model is that during carcinogenesis a field of premalignant epithelium precedes the development of cancer. This field of cells with genetic alterations develops and expands by lateral displacement in a process of 'Darwinian' clonal selection at the expense of uninvolved tissue. The majority of these fields are not visible and do not give any symptom despite their sometimes large dimensions. In the course of time, a cell within the field may develop into a cancer cell as a result of a series of crucial genetic hits and this cell may evolve into an invasive carcinoma. When after diagnosis and excision of the primary HNSCC a field remains, there is the continuous threat for relapse, at the site where the primary tumour was located or in the vicinity. According to the presently applied clinical criteria this can be designated 'local recurrence', if it develops within 3 years and within 2 cm in relation to the primary carcinoma and 'second primary tumour' (SPT), if these criteria are not met.[14] From the molecular point of view, it is appropriate to designate this type of relapse 'second field tumour' (SFT).[13] Previously, we studied the origin of local recurrences, clinically defined as above, and found that approximately half of the cases were an SFT and the other half was the result the outgrowth of MRC.[9,15] Not only from the conceptual, but also from the therapeutic point of view, discrimination between a field and MRC as a risk for the development of recurrent cancer may be important. MRC may be cured by post-operative radiotherapy or re-resection. When a field-at-risk is demonstrated, the situation is less clear. Surgery is not a real option, because of the large dimensions of the field and the fact that the majority is not visible to the naked eye. Radiotherapy may not be indicated for preneoplastic lesions and may even be contra-indicated, since in theory it might accelerate the carcinogenic process. A more intensive surveillance during follow-up may be the best option for this patient group.

The notion that local relapse is sometimes designated 'SPT' on clinical grounds, suggests that the clinical occurrence is considerably higher than the 20% incidence of clinically defined local recurrences. Regardless the mechanism underlying local relapse, important information can be found in the surgical margins of the resection specimen.

Standard histopathology of surgical margins

Before determining the additional role molecular studies might have, the dilemmas that emerge when the present standard procedures are applied, warrant discussion. We refer to the routine histopathological protocols that are en vogue in most larger European cancer centers. According to these protocols thorough assessment of all surgical margins, both the mucosal and deep, is required. Slices of about 4 mm covering ideally all margins of the specimen are made, and the most 'patient-adjacent' section is first examined.[16] In histopathological assessment of the margins, proper orientation must be guaranteed. The deep margin is investigated in one or more central sections of the tumour, depending on the macroscopic closest distance between tumour and deep margin, and is reported separately.

During routine examination the distance of the squamous cell carcinoma to the deep or mucosal margins is measured. There is more or less consensus on the classification of the tumour-margin distance nowadays.[16–18] 'Clear' or 'clean' margins indicate that there is a distance of over 5 mm between the carcinoma and the margin, though some authors have proposed 3 or 10 mm. 'Involved' means that there is cancer in or within 1 mm of the margin, and as a consequence it is highly likely that cancer has remained in the patient. Finally, there is a grey area in between that is known as 'close', which refers to distance of 1–5 mm between the tumour and the margin.

Most publications demonstrate the relation between margins, involved or close, and a worse prognosis. Involved margins result in a shorter disease free survival,[5,7,19–24] and a shorter overall survival.[25–28] Nevertheless, there are also investigators who have failed to find the importance of an involved margin.[29,30] The application of post-operative radiotherapy may explain the findings in these two latter publications to a certain extent, but it remains to be solved why this treatment has not influenced the outcome in the majority of the other studies. The presence of close margins has prognostic significance and was reported to be associated with a shorter recurrence-free survival[5,20,22,31,32] and a shorter overall survival.[25,27]

The implications of the presence of epithelial dysplasia have not been clarified as yet. A survey among 476 American head and neck surgeons indicated that dysplasia in the margins is often not a reason to adapt the therapeutic planning.[18] There is a scarcity of literature regarding the possible prognostic effect of dysplasia in the margin on the risk of local relapse. Slootweg et al. noted a higher rate of SPT, whereas the percentage of local recurrences was not increased.[5] In a small study population Weijers et al. also noted a higher local relapse rate in case of dysplasia in the margin.[33] Another Dutch study showed that the presence of any grade of dysplasia is not associated with local relapse.[31] In theory, the grading of epithelial dysplasia in the surgical margins may have value in assessing the risk for developing local recurrences.[34] However, risk assessment by such histological grading is hampered by subjectivity and low reproducibility,[34,35] limiting its value for predicting the risk for the individual patient.[36]

Molecular studies: potential markers

A list of studies on molecular markers with potential to identify tissue at risk for local relapse is depicted in Table 1. Most of the studies have used formalin-fixed paraffin-embedded (FFPE) margins, but also extra biopsies and brushed cells were taken from tumour-adjacent normally looking mucosa. Various techniques, all with their pro's and con's, have been used. Protein markers were detected by immuno-histochemistry, as was done for *TP53*, *CCDN1*, p16, *CHEK2* and *LAMA5*. This technique is relatively easy to perform, but has problems with objectivity of the scoring, setting cut-off points and reproducibility. DNA-based techniques are based on DNA copy-number changes (such as interphase-FISH, CGH, DNA-ploidy and MLPA; see for explanation Table 1), promoter-methylation (of the genes *MGMT*, p16, *DAPK1*), allelic imbalance with microsatellite markers or mutation analysis (*TP53*). Compared to immuno-histochemistry, measurement of DNA-based markers has a higher level of objectivity due to a better reproducibility and standardized cut-off levels (e.g. presence or absence of a