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Review article

Molecular markers in laryngeal squamous cell carcinoma: Towards an integrated clinicobiological approach

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Abstract

Of the most frequent malignancies in the United States, cancers of the larynx and of the uterine corpus are the only ones not to show an increase in 5-year survival rates over the last 30 years. The increasing use of chemo- and radiotherapy and conservative surgery to preserve organs and their functions has probably led to a better quality of life in patients with laryngeal cancer, but has definitely failed to improve survival, which remains the primary aim. In our opinion, to reduce laryngeal cancer-related mortality, a change in clinical approach is required. We have reviewed the literature on the potential role of molecular markers in the clinical management of laryngeal cancer. We believe that some of the most significant biological markers might be integrated with the evaluation of behavioural risk factors, clinical TNM staging and histopathological grading for a novel clinicomolecular approach to laryngeal cancer. We foresee the use of the most promising biological markers in the phases of prevention, diagnosis, prognostic assessment and drug design.

Keywords: Larynx; Head and neck; Cancer; Biomarkers; Clinical practice

1. Introduction

Laryngeal squamous-cell carcinomas (LSCC) comprise the vast majority (≈96%) of laryngeal malignancies [1]. Although the larynx is part of the head and neck, it has several clinical and molecular peculiarities. The American Cancer Society classifies the larynx as part of the respiratory system, separate from the oral cavity and pharynx [2]. The male/female ratio for the incidence of laryngeal cancer is much higher than in other parts of the head and neck [2]. Differences in chromosomal pattern and carcinogenic progression between LSCC and other head-and-neck squamous-cell carcinomas (HNSCC) have been detected by comparative genomic studies [3]. In particular, p53 is normally expressed in LSCC more frequently and the *p53* gene has a mutation pattern more similar to that in lung SCC than in other HNSCC [4].

In the United States, LSCC is estimated to account for almost 0.8% of all new cases of malignancy, with an incidence of about 10,000 cases per year, and to have caused 0.6% of all cancer deaths in 2004 [2]. Most of these tumours originate in the glottis (more than 60%) and supraglottis; the subglottis is an extremely rare site of origin (less than 5%) [1]. The estimated incidence of cervical lymph-node metastases with no obvious primary site ('occult T') is from 3% to 9% [5,6], and some of these might reasonably have a laryngeal (especially supraglottic) origin. Practically all patients (≈95%) with LSCC have a history of tobacco smoking, which increases risk in a dose-dependent way.

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In LSCC patients, second primary tumours (SPT), classically explained by the process of 'field cancerisation' [7], arise at an annual rate of 4–7% regardless of the initial treatment [8–10]. About 10% of LSCC patients have a history of other cancers and about 5% have a synchronous cancer [1]. Most common SPT are of the upper aerodigestive tract, with the lung being the most frequent site of origin; prostate and colon are the most frequent sites outside the upper aerodigestive tract. Conversely, LSCC is the new primary cancer with the highest standardised incidence ratio overall among patients with SCC of the lung [11]. Following the diagnosis of a first laryngeal malignancy, the median time interval to SPT is significantly lower when it develops in the upper aerodigestive tract than when it arises in other sites [9], suggesting that different pathogenetic and risk factors are involved. The probability of developing SPT (both synchronous and metachronous) seems to be increased by continued tobacco use and influenced by the site of the first-diagnosed malignancy [8,12].

The standard options for treatment of LSCC are surgery, radiotherapy, chemotherapy, or a combination of these. Radiotherapy is used in more than 70% of patients, surgery in about 55% and chemotherapy in about 10%. It is widely accepted that most early-stage LSCC can be adequately treated with single-modality therapy, whether surgery or irradiation, with a 5-year local control of 85–98% [10,13,14]. A multimodality approach based upon a combination of surgery and irradiation is the most common treatment for stage III and IV disease [1].

Of the most frequent malignancies in the United States, cancers of the larynx and of the uterine corpus are the only ones not to show an increase in 5-year survival rates over the last 30 years [2]. In LSCC, we can identify several potential reasons for this failure.

- The increasing use of chemo- and radiotherapy and more conservative surgery to preserve organs and their function [1] may have led to a better quality of life for LSCC patients, but has clearly failed to improve survival, which remains the primary aim.
- The majority of patients with LSCC (more than 60%), especially glottic cancers, present with early-stage disease, and early diagnosis remains the best predictor for survival. There is a reported increase in patients diagnosed with stage IV cancers, particularly in the supraglottis. Less than 1% of patients with LSCC are asymptomatic at presentation [1]. As the sensitivity of the most frequently used diagnostic procedures, though relatively high, is not absolute [97% for direct laryngoscopy, 90% for indirect laryngoscopy, 80% for CT of the primary site [1]], a number of LSCC patients may escape the first diagnostic approach. For most of its natural course, LSCC is clinically silent and even histologically occult, so no current protocols for clinical screening can sufficiently anticipate tumour detection.
- The TNM classification is in some cases inadequate. For example, regrouping cases in stages III and IV into locally advanced disease vs. regional metastasis appears to predict survival better [1].
- The clinical TNM often underestimates the extension of the disease when compared with the 'real' pathological TNM, which places a significantly higher proportion of tumours in the advanced-stage (III or IV) group [1]. Clinical methods are inaccurate predictors of pathological findings and may underestimate disease extension and macroscopically uncertain margins. Therefore, the TNM cannot be adequately evaluated in patients treated exclusively by radiotherapy, in the absence of a surgical specimen. After irradiation, it can become very difficult to assess data obtained by imaging and endoscopy for the diagnosis of both minimal residual disease and early recurrence.
- Despite the multiplicity of clinical prognosticators, the only consistent clinical predictors for disease control and disease-specific survival in LSCC are T and, to a greater extent, N [8,10,15]. The prognostic stratification of LSCC patients is inadequate since similar patients, affected by tumours with similar clinicopathological features and undergoing the same treatment, may differ widely in prognosis, probably due to the extreme biological heterogeneity of LSCC, which contributes to the lack of consistency in treatment planning.
- This lack of consistency is exemplified in the management of cervical lymph nodes, which is an important part of the overall treatment strategy, especially for supraglottic tumours. Surgery remains the mainstay of neck treatment, since it provides comprehensive clearance of all grossly enlarged lymph nodes and allows us to obtain accurate histological information about micrometastases in the clinically negative neck. Nevertheless, while the indications for comprehensive surgical clearance of the neck for clinically palpable metastatic lymph nodes (cN+) are obvious, those for elective selective treatment of the N0 neck appear less clear [8].
- SPT are the primary cause of death in patients with early-stage tumours [8,9,12], in particular those with early glottic cancers [10] characterised by a high rate of locoregional control. In spite of all the recent interest in developing effective chemopreventive drugs, effective measures that help people to stop smoking (e.g. the transdermal nicotine patch) remain the only means of reducing the incidence of SPT [12].

In our opinion a different approach to LSCC, based on genetics and molecular biology in addition to the clinical and histological approach, is required to overcome

these obstacles and to reduce cancer-related mortality in LSCC patients.

2. Potential clinical application of molecular markers: when? why? which?

Although the best-known risk factors, clinical TNM and histopathological grading will retain their value, it is now possible to acquire biological information about host and tumour to optimise the management of LSCC.

Systematic study of biological markers might be integrated into clinical practice in the phases of prevention as 'molecular epidemiology', of diagnosis as 'molecular diagnostics', of prognostic assessment and treatment selection as 'molecular characterisation' [16], and of the synthesis of new drugs as 'molecular targeting'. We have outlined this scheme in Table 1.

2.1. Molecular epidemiology

The best-established risk factors for LSCC are behavioural, e.g. cigarette smoking and heavy drinking. Primary prevention can be easily obtained by abandoning adverse habits, but not all LSCC patients have a history of behavioural risk factors or clinically evident precancerous lesions. Molecular epidemiology should help us to recognise patients and/or areas of laryngeal mucosa with a high susceptibility for developing LSCC, and possibly to identify molecular targets for effective secondary prevention (chemoprevention).

The incidence of genetic alterations in dysplastic, premalignant lesions is greater than half that found in invasive HNSCC [17]. The latency between carcinogen exposure and the appearance of malignancy may be as long as 25 years, so important molecular alterations should be detectable in affected mucosa many years before an invasive phenotype is produced, and presumably some of these will be more strongly associated with progression toward carcinoma. Several cellular alterations have been tested in clinical studies as potential markers of commitment to transformation (molecular histopathology), both in premalignant lesions and in apparently healthy mucosa [18–21]. Markers of commitment could help in the early diagnosis of malignant transformation by stringent follow up of high-risk mucosal areas, and in timely secondary prevention by immediately evaluating its effects at the molecular level [19].

The p53 pathway is of great interest in this aim. Alterations in p53 status have been extensively studied in tumour cells, in precancerous lesions and in apparently healthy mucosa of HNSCC patients, in the hope of verifying the intriguing hypothesis that they might predict the development of SCC (also of SPT) [18,22–24]. Mutations of *p53* have also been evaluated to establish whether multiple primary tumours have a mono- or polyclonal origin [25,26]. Although no definitive conclusions have yet been drawn about these fundamental issues, a coherent model is now beginning to emerge [27], and the use of p53 alterations as a marker to identify 'condemned mucosa' remains an intriguing, if still hypothetical, possibility. In LSCC, *p53* expression is altered less frequently, with a mutation pattern different from that of other HNSCCs (and more similar to lung SCC) [4]. If we assume that the p53 pathway is impaired in some way in every malignant epithelial neoplasm, then alternative mechanisms of inactivation could be particularly relevant in LSCC [4]. Degradation mediated by other cellular proteins, such as mdm2 [28], or by human papillomavirus (HPV) E6 oncoprotein [29], may represent two such alternative pathways leading to loss of p53 function.

Chromosomal alterations, such as 9p21 loss, are prevalent and early events in carcinogenesis, and proposed targets for preventive strategies [17,19]. The overexpression of the epidermal growth factor receptor (EGFR) [18], alterations in cyclin D_1 (in the earliest phases, overexpression, and later *CCND1* amplification) [18,30] and high telomerase activity [31] are early events, and may be potential markers for the prediction of neoplastic progression.

Individual susceptibility to LSCC may derive from environmental or genetic factors. A genetic predisposition to the development of LSCC is highly probable. Intrinsic sensitivity of cells to mutagens such as bleomycin is a biomarker of HNSCC susceptibility [32]. Polymorphisms of carcinogen-metabolising enzymes known to be involved in the metabolism of carcinogens found in tobacco smoke are relatively common in most populations. A growing body of evidence suggests that many of these genetic polymorphisms are associated with the risk of developing cancers of the aerodigestive tract [33–35]. In particular, the risk of developing LSCC has been evaluated in relation to polymorphisms of genes encoding for arylamine *N*-acetyltransferases [36], human OGG1 DNA repair enzyme [37], CYP1A1, XRCC [38] and glutathione *S*-transferases, with controversial results [39,40]. These and other detoxifying enzyme genes might be evaluated in the future to assess susceptibility to environmental carcinogens and thus the risk of developing LSCC in association with tobacco smoking.

2.1.1. Human papillomavirus infection

Among environmental factors, previous exposure to HPV 16 correlates with a marked increase in risk for oropharyngeal cancer [41,42]. Several reports suggest a role for high-risk HPV genotypes ([16,18,31,33] and presumably others with a more selective tropism for the head and neck) in oral [43] and in laryngeal [44–49] carcinogenesis. The frequency of HPV infection in LSCC varies widely (3–85%) among different studies, also depending on the detection technique [44–50].

Table 1
Molecular markers as evaluated in patients from a single institution; most promising phases of clinical application are shown in bold type

Molecular marker	Function in the normal cell	Alterations in tumour cells	Clinical phase of application
Epidermal growth factor receptor (EGFR)	Receptor for growth factors (as TGF and EGF) with tyrosine kinase activity. Upstream activator of MAPkinase pathway and of other pathways involved in cell growth, cell migration, block of apoptosis (Fig. 1).	Frequently and early overexpressed in LSCC, mainly by post-translational mechanisms. At present the most reliable biological marker for molecular characterisation. Marker of aggressiveness [79,80] and of invasiveness [81].	Molecular epidemiology, **Molecular characterisation**, **Molecular targeting**
Telomerase activity	A low telomerase activity, required for telomere lengthening and autonomous replication, can be detected in haematopoietic tissue, in some immune cells (activated lymphocytes), in basal epithelial layers. Absent in most non-transformed differentiated cells.	Present, often at high levels, in most laryngeal cancer cells. It can at least partly depend on *h*-TERT gene (coding for catalytic subunit of telomerase) overexpression [31].	Molecular epidemiology, Molecular diagnostics, Molecular targeting
Overexpression and amplification of cyclin D_1 gene (*CCND1*)	Cyclin D^1 gene transcriptional activity normally strictly depends on mitogen stimulation, and leads to cell commitment to mitosis through START checkpoint.	An early *CCND1* overexpression is often detectable without evidence of gene amplification; it can be used for molecular epidemiology but it seems to retain a lower prognostic value, if compared with *CCND1* amplification, a marker of aggressiveness in LSCC [92].	Molecular epidemiology, Molecular diagnostics, **Molecular characterisation**
Cathepsin D	Lytic enzyme active in extracellular matrix rearrangement.	An overexpression is often detectable in tumour cells, where it seems to contribute to invasiveness [94].	Molecular characterisation
Human papillomavirus (HPV)	Normally absent in cells, HPV affect epithelia with mucosal or epidermal tropism according to genotype.	Important oncosuppressors such as p53 and pRb are inhibited and degraded by HPV oncoproteins. In turn, overexpression of oncogenes such as EGFR and cyclins A and B is induced [44,55–57].	**Molecular epidemiology**, Molecular diagnostics, Molecular characterisation
Type II oestrogen-binding sites (EBS)	Normally present in laryngeal mucosa.	Type II EBS may at least partly mediate tumour growth inhibition by tamoxifen and quercetin; possible targets for chemoprevention and therapy [110].	Molecular characterisation, Molecular targeting
S100-A2 Ca^{2+} binding protein	Increasing levels of expression during differentiation of squamous epithelial cells; absent in basal layers.	Underexpression in cancer cells, inversely proportional to tumour differentiation. Starting from data on NSCLC a role as a real oncosuppressor has been hypothesised [99]	**Molecular characterisation**
Methyl-*p*-hydroxyphenyllactate esterase (MEPHLase) activity	Enzyme involved in the metabolism of methyl-*p*-hydroxyphenyllactate, ligand of type II EBS, with a role in growth and differentiation of several tissues (breast, uterus), normally expressed in larynx.	In LSCC a low activity is associated with poor differentiation, and shorter overall survival and metastasis-free survival [111].	Molecular characterisation
Type 2 cyclo-oxygenase (Cox-2)	Enzyme involved in arachidonic acid metabolism and autacoid synthesis, induced by various stimuli in several cell types. Inhibited by FANS.	Cox-2 activity seems to promote tumour neoangiogenesis. Nevertheless, evidence exists that low Cox-2 expression indicates poor differentiation and higher aggressiveness and invasiveness [65].	Molecular characterisation, Molecular targeting
Galectin-3	Galectin-3 is a pleiotropic carbohydrate-binding protein participating in a variety of cell processes, and mediating cell-to-cell interactions	Galectin-3 expression seems positively associated with tumour keratinisation and histological grade. A significant correlation was found between galectin-3 tumour positivity and longer metastasis-free and overall survival in LSCC patients [98].	**Molecular characterisation**

Real-time, quantitative polymerase chain reaction to E6 and E7 regions of the high-risk genotypes so far seems the most promising tool for clarifying the epidemiological relevance of HPV in LSCC [51].

In experimental models, exposure to tobacco-related mutagens produced substantially more genetic alterations in HPV-immortalised human keratinocytes than in normal keratinocytes [52–54]. Most epidemiological studies suggest that high-risk HPV infection substantially increases the risk of HNSCC development in smokers [42–44]. Therefore, HPV DNA may be searched for as a marker of susceptibility to environ-

mental carcinogens and, in combination with behavioural (and also genetic) risk factors, might help identify a group of high-risk individuals to submit to strict follow up, and to primary and secondary (chemo-) prevention [55].

Conversely, if a subgroup of LSCCs is aetiologically linked to HPV infection, it is likely to be rather homogeneous. In fact, HPV has been associated with peculiar molecular characteristics such as *CCND1* gene amplification [55,56], EGFR overexpression [57], and a lower rate of *p53* gene mutations [42]. HPV infection might guide tumour progression toward a typical molecular and histological pattern, and consequently determine a characteristic, homogeneous clinical behaviour. For example, HPV-positive carcinomas reportedly have a better prognosis (at least in the oropharynx) and are more radiosensitive [42]. These possibilities should be addressed in the future: gene-expression profiling by cDNA microarrays might be an appropriate tool for defining any molecular pattern linked to HPV infection, which would have particular relevance to the aim of better prognostic stratification and therapeutic planning (molecular characterisation).

In the phase of diagnosis, the presence of HPV genomic material in serum DNA of HPV-positive HNSCC patients after treatment might be used as an early marker of local and/or regional recurrence [58].

2.1.2. Chemoprevention

Chemoprevention, defined as an attempt to reverse, suppress or delay the progress from normal mucosa towards invasive cancer [59], could be considered the final vision of molecular epidemiology. The larynx is a site of major interest for clinical trials with chemopreventive agents for at least three reasons:

1. The high incidence of SPT in LSCC patients, in particular those with early glottic cancer [10,12] (see Section 1).
2. The high incidence of premalignant lesions (i.e. leukoplakia, erythroplakia) in laryngeal mucosa of particular populations (heavy smokers and drinkers). In particular, laryngeal leukoplakia is a potentially malignant lesion with a transformation rate ranging from 3% to 30%, depending also on histology [60] and on molecular pattern [19]. Proposed clinical attitudes range from 'watchful waiting' to surgical resection, but the latter does not appear effectively to prevent transformation in other areas of apparently healthy mucosa in the 'field of cancerisation' [60].
3. Laryngeal mucosa is easily accessible and it is therefore relatively simple to diagnose premalignant lesions and then to assess the response after chemopreventive therapy [60], allowing also an evaluation at a molecular level [19].

These considerations have been the basis for interest in developing effective, non-toxic chemopreventive drugs to reduce the risk of developing second cancers, but agents proposed so far have not been clearly effective in precancerous lesions, or on the development of second malignancies. Retinoids, though having considerable toxicity, have been suggested as chemopreventive agents and encouraging results are reported in the treatment of laryngeal precancerous lesions [61]. Nevertheless, in EUROSCAN, the largest clinical trial so far, a 2-year supplementation with retinyl palmitate and/or *N*-acetylcysteine gave no benefit for survival, event-free survival or SPT to patients with HNSCC or lung cancer [62]. Thus, the primary goal of decreasing the incidence of SPT in patients treated with curative intent for early-stage disease is still far from being obtained.

Based on results in colorectal adenocarcinoma [63], selective cyclo-oxygenase-2 (COX-2) inhibitors have been proposed as chemopreventive agents in precancerous lesions of the head and neck [64]. COX-2, an enzyme that catalyses the synthesis of prostaglandins, is overexpressed in a variety of premalignant and malignant conditions, including HNSCC [64]. In our experience, such overexpression occurs early in carcinogenesis of the larynx and tends to be lost in advanced and high-grade tumours [65]. Therefore the use of COX-2 inhibitors in the phase of prevention seems rational.

Our recent findings suggest a role for hypofolataemia as a risk factor for HNSCC [66,67] as for colon cancer [68], which would at least in part account for the high incidence of colon SPT in HNSCC patients [9]. More importantly, folate status may be considered a novel target for primary (also dietary) and secondary (chemical) prevention. Folate has no known toxic effects and was apparently effective, in association with vitamin B_{12}, in inducing the regression of precancerous lesions such as bronchial squamous metaplasia [69,70]. In an animal model using beagles treated with *N*-ethyl-*N*-nitrosoguanidine, folate had a strong protective role against the development of gastric cancer [71]. A chemoprevention protocol with folic acid, under strict histological and clinical follow up, is in progress at our institution in a group of patients with laryngeal leukoplakia, with encouraging preliminary results [67].

2.2. Molecular diagnostics

Molecular diagnostics should help us to:

1. diagnose biological transformation, even with negative histology;
2. detect extremely early neck node involvement (occult metastases);
3. assess precisely, even in the absence of surgical specimens, the local and regional spread of the tumour;

4. detect minimal residual disease at the margins of surgical resection and at the primary site after irradiation;
5. diagnose extremely early recurrences.

The clinical goals of molecular diagnostics are earlier initial or salvage treatment and safer oncological effectiveness with better functional results. The detection of minimal residual disease and of early recurrences and metastases by molecular markers would be particularly useful after radiotherapy, when for several months radiation damage makes the evaluation of treated sites more difficult, often with fatal diagnostic delay.

The unidentified primary (occult) HNSCC presenting only as a cervical lymph-node metastasis despite thorough examination should be another target for molecular diagnostics. Histopathologically benign mucosa of the upper aerodigestive tract may harbour foci of clonal, preneoplastic cells that are the site of origin of genetically related, metastatic HNSCC. The use of multiple biopsies aiming to map, for example by microsatellite analysis, the mucosal sites that harbour these clones may be a useful tool [6].

The perfect marker for molecular diagnostics should be present in all LSCCs, easily detectable even in histologically occult cases (high sensitivity), indicative of non-reversible passage from severe dysplasia to cancer (capable of differentiating precancerous lesions from early carcinomas) and thus always absent in non-cancerous mucosa (high specificity). No biological marker with these characteristics has yet been described; all molecular features proposed so far only approximate such an ideal.

One of these is eIF4E overexpression, which can be demonstrated in practically all LSCCs and whose presence in histologically tumour-free surgical margins predicts recurrence with discrete specificity and good sensitivity [72]. Loss of p16 expression is one of the most frequent molecular abnormalities in HNSCC [73] and seems be important in laryngeal carcinogenesis [74,75], but its clinical potential in LSCC requires further evaluation. *p53* mutations appear to predict local recurrence when detected on surgical resection margins [76]. An alternative cytogenetic approach has been proposed by Califano et al. [17].

2.3. Molecular characterisation

Molecular characterisation by the study of predictive molecular factors aims to define homogeneous groups of patients for prognostic stratification and treatment selection. Although a plethora of studies have sought to evaluate their potential, no molecular marker yet contributes to clinical decision-making. Based on our experience (Table 1) with a large patient population enrolled from a single institution, homogeneous for site (larynx) and with a long follow up, we attempted to outline how molecular markers might be integrated with standard TNM and histological grading (clinicopathological parameters).

The perfect marker for molecular characterisation of LSCC should not be constantly present in malignant cells but invariably associated with precise biological features and predictable clinical behaviour, and easily detected by a standard, reliable and simple assay on a small sample such as from a biopsy. No such marker yet exists. Recently, cDNA microarrays, a powerful tool from which large amounts of genetic information can be obtained, have been used for an initial tentative molecular classification in HNSCC, based on patterns of global gene expression [77,78]. This method can only be applied to frozen tissues, because RNA is destroyed during formalin fixation, and a frozen tumour bank combined with a strong clinical database and complex statistical capacity would be required to make full use of this expensive technology. Searching for three or four well-defined biological markers with more reliable assays might allow us to classify tumours as positive (Mc+) or negative (Mc−) for molecular characterisation. We should try to assess at least some of the main biological features of tumours, such as aggressiveness, invasiveness, radio- and chemosensitivity. We define tumour aggressiveness as the tendency to local disease progression, and invasiveness as the tendency of tumours to metastasise. These characteristics, intrinsic to a given tumour and revealed also by some of the molecular markers studied by our group (Table 1), have a large influence on prognosis and should guide therapeutic decisions. TNM staging could then become TNM-Mc staging, resulting in better prognostic stratification of patients and the selection of the most suitable, individualised treatment. It would prevent the overtreatment of Mc− patients and, most importantly, the undertreatment of Mc+ patients, which has probably contributed to the cited failure to improve prognosis in LSCC over the last 30 years.

- In Mc+ tumours, larger resection margins could be planned with a more ablative approach to primary tumour, since conservative surgery is too risky in these cases. In cN0 patients, a more aggressive approach in the neck would involve elective bilateral dissection or elective irradiation. In cN1 patients, comprehensive ipsilateral neck dissection, with elective selective contralateral neck dissection, might be possible. Adjuvant radiotherapy could be advised also in tumours with histologically negative resection margins, and/or in stage pN1 without extracapsular spread.
- In Mc− tumours, integrated staging might allow safe indications for conservative surgery and thereby functional preservation, lowering at the same time

the risk of failures. In the neck 'a wait and see' approach to cN0 tumours and selective rather than comprehensive ipsilateral dissection, without elective contralateral dissection in cN1 tumours, could be justified.

2.3.1. Characterising molecular markers

Among the markers evaluated so far, some have appeared potentially reliable and suitable from a clinical perspective.

There is strong evidence of a role for EGFR expression (and, to a lesser extent, for its ligand, transforming growth factor-α) in predicting prognosis, because it adversely influences overall relapse-free and metastasis-free survival in LSCC. EGFR retains a strong predictive value independently of treatment (surgery, chemotherapy and radiation) [79–84], which makes it, in our opinion, the most reliable prognostic molecular marker at present. Furthermore, EGFR overexpression seems to predict both chemo- and radioresistance [83,85].

Alterations of p53 protein expression and mutations of the *p53* gene have been extensively studied as predictors; changes in p53 are proposed independent predictors of recurrence in LSCC [28,86], but this prognostic value is controversial [4], especially in surgically treated patients [87]. p53 overexpression, detected by immunohistochemistry (IHC) in an high percentage of LSCC [88], appeared to correlate well with *p53* mutation [89], but a recent study has shown significant discrepancies between p53 IHC and genotyping data [90]. *p53* gene mutation has been suggested as more reliable than IHC overexpression for characterisation and reportedly predicts the response to radiotherapy in LSCC patients [90]; this observation is consistent with the biological role of p53, which mediates apoptosis associated with DNA damage.

A separate prognostic role has been described for cyclin D_1 protein overexpression [91] and *CCND1* gene amplification [92], with an impact on relapse-free and overall survival in HNSCC. Studies on breast tumours and also on HNSCC have shown that *CCND1* amplification, rather than protein overexpression, might have prognostic value [93].

There is recent evidence that the degradation of extracellular matrix by metalloproteases and cathepsin D is important in tumour growth, invasion and metastasis, as well as in tumour-induced angiogenesis [94–96].

Laminin-5 [97], galectin-3 [98], Cox-2 [65], as markers of epithelial differentiation, might have predictive value, specifically by integration with classical histological evaluation. S100 A_2 has recently been described not merely as a differentiation marker but as an actual oncosuppressor with a prognostic significance stronger than simple histopathological grading [99].

Various problems affect the clinical application of molecular markers for tumour characterisation. First, the perfect marker has yet to be demonstrated; in particular the detection assays must be practical and reliable, and should be widely available. The inconsistency of assay methods for most factors studied, and patient and treatment heterogeneity, all detract from an ability to draw definitive conclusions. We need to evaluate every molecular marker proposed for clinical practice both by a meta-analysis of published data and by multidisciplinary and multicentre clinical trials.

2.4. Molecular targeting

The use of cellular tumour markers as therapeutic targets is being studied. The goal is to block specific pathways involved in the carcinogenic process and/or in tumour pathogenicity (aggressiveness, invasiveness), and to restore effective oncosuppression.

It is evident that the EGFR pathway is a good target for therapeutic interventions in LSCC. Several approaches have been assessed for their ability to interfere with EGFR function and might be useful for anticancer therapy. These approaches include the use of monoclonal antibodies (MAb) against the extracellular ligand-binding domain, ligand–toxin conjugates that kill target cells following endocytosis, tyrosine kinase inhibitors that inhibit ligand-induced EGFR activation, and antisense oligonucleotides against EGFR mRNA [100,101] (Fig. 1).

Various blocking MAb have been developed against the EGFR; their clinical efficacy is related to the inhibition of receptor tyrosine kinase activity and thus of cell-cycle progression, angiogenesis, invasion and metastasis (by reducing lytic enzymes). Some MAb also influence the induction of apoptosis, cell-mediated immunity and radio- and chemosensitisation. Anti-EGFR MAb have already been tested in phase II and III studies on humans, alone or in combination with conventional therapies such as radiotherapy and chemotherapy, with very promising results. In particular, there is a large body of evidence that anti-EGFR MAb have impressive activity when combined with radiotherapy, and reverse resistance to chemo- and radiotherapy in advanced HNSCC [102,103].

The tyrosine kinase activity of the EGFR is required for the biochemical responses induced by this receptor [104]. Over the last decade, research has produced a variety of inhibitors of EGFR tyrosine kinase acting intracellularly [105]. Some quinazolone derivatives specifically inactivate both the EGFR and ErbB2 in an irreversible manner ('pan-HER' inhibitors). The antiproliferative activity of ZD1839, a 'pan-HER' inhibitor, in combination with a number of cytotoxic drugs has been assessed against a variety of human cancer cell lines [106], with an enhancement of growth-inhibitory

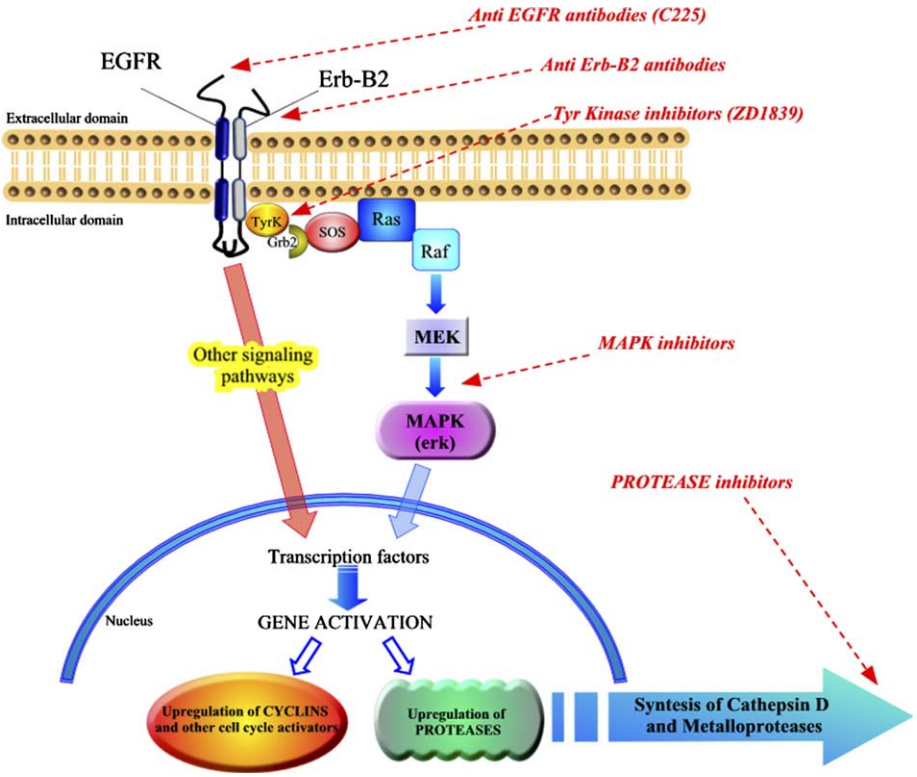

Fig. 1. A scheme of the epidermal growth factor receptor (EGFR) pathways with the steps targeted by therapeutic experimental approaches.

effects. Preliminary results suggest that ZD 1839 has promising antitumour activity also *in vivo*, particularly against non-small cell lung cancer [105].

Transient overexpression of the wild-type *p53* gene is considered to provide a potential molecular intervention strategy in various malignancies [107]. In HNSCC, the most used gene-delivery tool has been the recombinant adenovirus Ad-p53 [108]. A gene therapy approach using wild-type human *p53* has already been shown to induce apoptosis, radio- and chemosensitisation in cell lines [109], and the use of *p53* gene therapy in combination with radiotherapy or chemotherapy is surely a rational possibility. Another potential application for *p53* gene therapy is the treatment of dysplastic lesions, as *p53* mutations seem to occur early in head-and-neck carcinogenesis. The main problem, as for most such approaches, remains the absence of a really efficient, long-term gene-delivery system.

Conflict of interest statement

None declared.

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