Molecular Alterations in Nodal Metastases and its Primary Tumors in Squamous Cell Carcinomas of the Larynx

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Introduction and objectives: The successive acquisition of molecular alterations determines tumour progression. During this progression, the development of nodal metastases is one of the most important prognostic factors in laryngeal squamous cell carcinomas. The aim of this study is to analyze if, in these carcinomas, the molecular alterations in the nodal metastases are different from those present in the primary tumour.
Material and method: Paired samples of primary tumour and nodal metastases from 51 patients with squamous cell carcinoma of the supraglottic larynx were studied. Using immunohistochemistry, we analyzed the expression of p53, E-cadherin, FAK, annexin A2, and HIF-1α proteins. In addition, the apoptotic index (measuring activated caspase-3) and the degree of vascularization (identified by CD34 antigen expression) were also studied.
Results: A close correlation in the expression of the proteins studied was observed in the nodal metastases and the corresponding primary tumour, with the exception of HIF-1α expression and the degree of vascularization.
Conclusions: Most of the molecular alterations in the nodal metastases are already present in the primary tumour, suggesting that these alterations are early events in carcinogenesis.

Key words: Squamous cell carcinoma. Larynx. Metastasis. Molecular markers.

Alteraciones moleculares en las metástasis ganglionares y sus tumores primarios en los carcinomas epidermoides de laringe

Introducción y objetivos: Presentar alteraciones moleculares sucesivas determina la progresión tumoral. Durante esta progresión, el desarrollo de metástasis ganglionares es uno de los determinantes más importantes del pronóstico de los carcinomas de laringe. En este estudio se analizará si en estos carcinomas las alteraciones moleculares en las metástasis ganglionares difieren de las de su correspondiente tumor primario.
Material y método: Se estudian muestras apareadas de tumor y metástasis ganglionares de 51 pacientes con carcinoma epidermoide supraglótico. Se determina, mediante inmunohistoquímica, la expresión de las proteínas p53, E-cadherina, anexina A2, FAK, y HIF-1α, y además la actividad apoptótica (mediante la expresión de caspasa-3 activada) y el grado de vascularización (identificando los vasos por la expresión del antígeno CD34).
Resultados: Se apreció una marcada correlación en la expresión de las proteínas estudiadas en las metástasis y su correspondiente tumor primario, con la excepción de la expresión de HIF-1α y el grado de vascularización tumoral.
Conclusiones: La mayoría de las alteraciones moleculares en las metástasis ganglionares ya están presentes en el tumor primario, lo que indica que estas alteraciones suceden de forma temprana en la carcinogénesis.

Palabras clave: Carcinoma epidermoide. Laringe. Metástasis. Marcadores moleculares.

INTRODUCTION

The main route for disseminating head and neck squamous cell carcinomas is by metastasis to the regional lymphatic ganglia. In addition, the presence of metastatic disease in the cervical lymph node is the most important factor determining the treatment and prognosis for these patients.[1] It is therefore essential to identify the mechanisms involved

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in the development of these metastases if we wish to advance in the control of disease.

The process of metastasis is complex and requires a series of sequential steps with multiple interactions between the tumour and the host tissue: a cell or group of cells must leave the primary tumour, migrate through the adjacent tissues, invade the lymphatic or blood vessels, survive there, and still be capable of colonizing and proliferating in the remote lymphatic ganglia or other organs.[2,3] In order to carry out these steps, tumorous cells have to have a series of characteristics, acquired through successive genetic alterations. But it will also be necessary to bear in mind the interaction with neighbouring cells and adjacent structures.

In the conventional model of metastasis, metastatic potential would present late on in carcinogenesis as a result of selective alterations in a small group of cells within the mass of the tumour.[4] Nonetheless, genetic expression studies have shown that metastases show genetic profiles similar to that of the primary tumours from which they stem, indicating that the metastatic potential is acquired early on during carcinogenesis and is maintained during the progression of the tumour.[5] Even more, it has been shown that metastases can be predicted by the patterns of genetic expression present in the primary tumour.[6,7]

Reports recently published have described lymph node metastases of squamous cell carcinomas in the head and neck as showing genetic expression profiles very similar to those of the primary tumour.[8] Apart from allowing metastatic dissemination, this would indicate that the genes expressed in the primary tumour also have an important role in the survival and proliferation of cells during metastasis. The genes involved in carcinogenesis and metastasis, therefore, can be expected to control processes such as cell survival, proliferation, adhesion, and migration. The altered expression of these genes can be studied through their reflection in the expression of the proteins whose synthesis they control. In this study, we analyze the expression of several proteins involved in the processes potentially related to metastasis in a group of lymph node metastases and their corresponding primary tumours in order to determine whether there is any correlation between them. The proteins selected are p53 (linked to the control of cell proliferation and survival), E-cadherin (a key protein in cell adhesion), FAK (focal adhesion kinase, which allows survival regardless of the anchor to the extra-cellular matrix), HIF-1α (sub-unit 1α of the hypoxia inducible factor, which participates in the adaptation of cells to this situation), and annexin A2 (a protein related to cell differentiation and adhesion). Furthermore, the paper analyzes 2 phenomena occurring during carcinogenesis and very important for metastatic dissemination: angiogenesis and apoptosis.

MATERIAL AND METHOD

Patients

Samples were studied from 51 patients operated on between 1988 and 1994 for squamous cell carcinoma of the supraglottal larynx presenting metastases in the cervical lymph node. In all cases, these were primary tumours that had not previously received treatment. The patients were subjected to therapeutic surgery involving supraglottal laryngectomy or total laryngectomy plus bilateral lymph node removal in all cases. All patients were male, with a mean of 63 years of age (range, 46-81). All except 1 had a prior history of regular smoking and 44 were also alcohol consumers. Patients were classified according to the staging criteria of the International Union Against Cancer (5th edition). The clinico-pathological characteristics of the patients are given in Table 1. Samples of the primary tumour and corresponding lymph node metastases were obtained from the files of the pathology laboratory.

Immunohistochemical Analysis

The tumour samples in paraffin were sliced into 4 µm sections and placed on silicone slides (DakoCytomation). These sections were deparaffinized and hydrated as per standard techniques. Antigen recovery was effected by heating the sections in citrate buffer for 10 min in a pressure cooker. The staining reactions were carried out automatically at room temperature on a TechMate 1000 work station (BioTEK Solutions) in a single session for each antibody. The samples were placed in blocking medium (3% hydrogen peroxide) for 15 min and then reacted with the primary antibody at room temperature. The antibodies used are shown in Table 2. Immunodetection was done using the Envision system (Envision Plus, Dako) and with diaminobenzidine as chromogen. Tinction with hematoxylin during 1 min was the last step. Following tinction, the sections were dehydrated and placed on a slide using standard medium. The positive controls comprised samples of tissues that were known to express the study proteins. Negative controls with omission of the primary antibody were also included.

Table 1. Clinico-Pathological Characteristics of the Cases Studied

Characteristics	*Patients, n*
pT classification	
T1	4
T2	15
T3	22
T4	10
pN classification	
N1	19
N2	20
N3	12
Staging	
III	16
IV	35
Histological degree	
Well differentiated	18
Moderately differentiated	19
Poorly differentiated	14

Table 2. Antibodies Used in the Immunohistochemical Study

Antibody	*Clone*	*Company*	*Dilution*
p53	DO-7	DakoCytomation	1:200
HIF-1α	54	Becton-Dickinson	1:50
E-cadherin	36	Becton-Dickinson	1:2000
FAK	4.47	Upstate Biotechnology	1:250
Annexin A2	–	Zymed	1:400
Caspase-3	5A1	Cell Signaling Technology	1:200
CD34	QBEnd/10	Novocastra	1:50

Two of the authors (JPR and RC) studied these preparations at random, without clinical information. The primary tumours and the metastases were analyzed in different sessions. In all cases, in order to compare the primary tumour and the metastasis, a dichotomy was made between the degree of expression of the proteins analyzed, according to prior statistical studies and analyses. In the case of p53, the number of tumorous cells with tinction was quantified at an intermediate magnification (×100) and tumours were classified as positive, in line with prior studies,[9,10] when there were more than 10% of tumorous cells stained. HIF-1α was quantified in a similar way (percentage of tumorous cells with nuclear tinction); a cut-off was also set at 10% of stained cells.[11] In the case of E-cadherin, each sample was scored according to the intensity of the membrane tinction (0-4) and the percentage of tumorous cells stained (0%-100%). Both elements were multiplied together to give a total score between 0 and 400; cases were considered positive when the score was higher than the mean and negative if they scored less.[12] Annexin A2 was quantified by giving a score only for the percentage of tumorous cells with membrane tinction (0%-100%), since most of the cells, when positive, presented intense staining. In this case, samples were considered positive when the score was higher than the median. In the case of FAK, as all the tumorous cells in each sample showed similar staining, the tumours were classified into 3 categories according to the intensity of the cytoplasmatic tinction: weak, moderate, and strong staining. For statistical purposes, the cases with moderate and strong staining were classified as positive and those with weak staining as negative.[12] The rate of apoptosis was determined by analyzing the expression of activated caspase-3 protein; for quantification, the tumorous cells stained in 5 fields chosen at random at a magnification of ×400 were considered and cases were positive when they scored above the mean.[13] Angiogenesis was assessed by determining the vascularization of the tumours and their metastases; for this purpose, blood vessels were stained with anti-CD34 antibody and the number of vessels existing a magnification of ×200 was quantified in the 4 tumour areas with the greatest number of vessels stained ("hot spots"). The largest score for vessels from the 4 areas quantified was taken as the degree of vascularization.[14] Cases were classified as positive when they scored more than the median.

Some cases could not be assessed for all of the markers due to the poor quality of the tinction or an insufficient amount of tumour in the sample.

Statistical Analysis

Statistical analysis was performed using version 11.0 of the SPSS programme. In order to analyze the association between the expression of the proteins studied, the apoptosis rate and the vascularization in primary tumours and their corresponding metastases, the χ^2 test was used. A P value less than .05 was considered significant.

RESULTS

The expression patterns of the proteins analyzed in squamous cell carcinomas of the larynx have already been described in previous papers[11,15-18] and coincide with those found in this work. Four of the 5 proteins studied (p53, E-cadherin, FAK, and annexin A2) showed similar expression in the lymph node metastasis and the corresponding primary tumour (Figure); the statistical association was highly significant in most cases (Table 3). The only exception was the expression of HIF-1α. Of the other 2 parameters analyzed, the apoptotic index (as determined by the expression of activated caspase-3) also showed a significant correlation between the primary tumour and the metastases, whereas there was no correlation for the degree of tumour vascularization (Table 3).

The protein that showed the greatest correlation for expression between the primary tumour and metastasis was p53 (there was discrepancy in only 1 case). For the other proteins presenting a positive correlation (E-cadherin, FAK, annexin A2, and caspase-3), in only a few cases was the expression in the metastasis increased or diminished with regard to that of the primary tumour. In addition, in most of the cases where there was a discrepancy, it was noted that these cases had expression levels close to the cut-off point chosen for the dichotomization, therefore small variations in the expression between the primary tumour and the metastasis might explain the divergent results.

For the E-cadherin and annexin A2 proteins, it was also seen that, in those cases where there was concordance between the primary tumour and metastases, there was a predominance of cases with diminished or negative expression (67% and 65% of cases, respectively). In addition, whenever there was a discrepancy, the metastases also showed a predominance of negative cases (Table 3). Among the concordant cases of FAK expression and in the apoptotic index, those with increased or positive expression predominated (76% and 85% of cases, respectively). The expression of FAK in the metastases of discordant cases was also predominantly positive (Table 3). With respect to the expression of p53, there was practically the same number of positive as negative cases.

As for the expression of HIF-1α and the degree of vascularization, although the number of cases with concordance was also greater than those with discrepancy, there was no significant correlation. In the primary tumours,

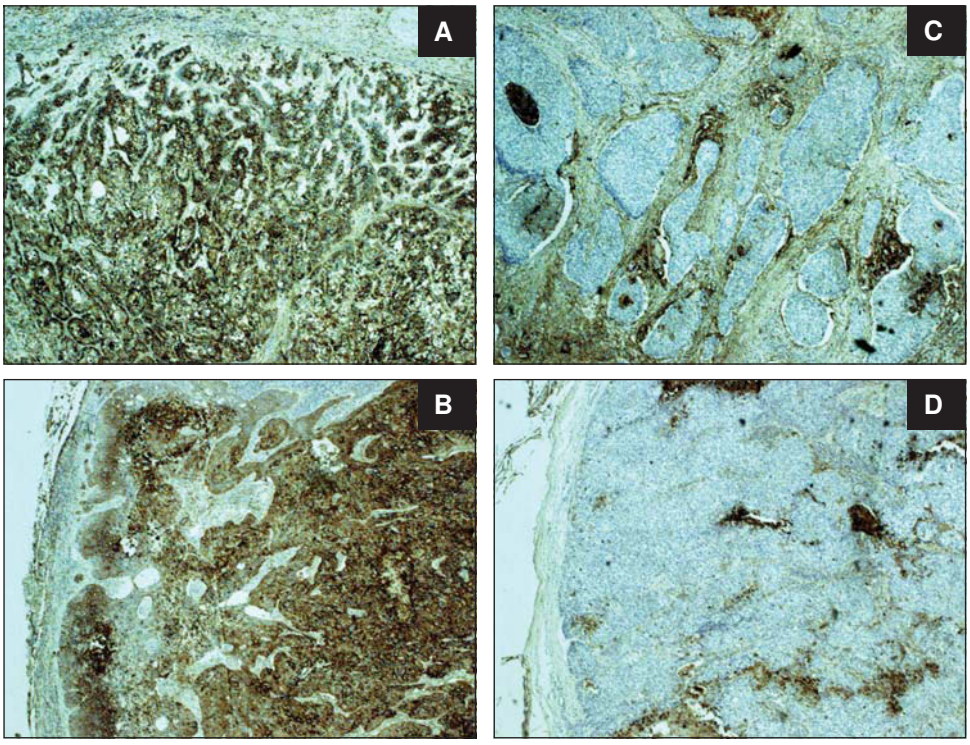

Figure. Example of the correlation of annexin A2 expression in the primary tumour and the metastases. Positive expression in the primary tumour (A) is reproduced in the corresponding metastasis (B), just as the negative expression in another tumour (C) also corresponds to that of its metastasis (D). Magnification, ×5.

the expression of HIF-1α was predominantly positive (68% of cases), but not in the case of the metastases (54% of cases). With respect to the degree of vascularization, there was no clear trend regarding a predominance of positive or negative cases (Table 3).

Table 3. Results of the Expression of the Proteins Analyzed in the Primary Tumour and Metastases

	n	*Concordance*		*Discordance*		*P* (χ^2)
Primary		+	–	+	–	
Metastasis		+	–	–	+	
p53	43	20	22	1	0	<.001
E-cadherin	46	13	27	4	2	<.001
FAK	46	26	8	5	7	.014
ANXA2	29	8	15	6	0	<.001
Caspase-3	23	17	3	2	1	.031
HIF-1α	44	18	8	12	6	

DISCUSSION

It is currently known that the genetic expression profiles in metastases are very similar to those in the primary tumours from which they originate, as has been shown in breast carcinomas and head and neck squamous cell carcinomas.[5,8] This indicates that the genetic alterations conferring the capacity to metastasize are acquired at an early stage of carcinogenesis, and that the primary tumour already presents the alterations allowing the metastatic cells to survive and proliferate in an alien setting. In addition, since the genetic alterations allowing metastases can be detected in the primary tumour, it is possible to use them to identify patients with a high risk of presenting metastasis.[7]

As the altered expression of genes does not always have the same repercussions on the expression of the proteins they encode for (for example, there may be post-transcriptional or post-transductional regulation), we have used in this study the detection of protein concentrations to compare their expression in the lymph node metastases and the corresponding primary tumour. The selected proteins are involved in the regulation of processes potentially linked with metastatic dissemination, such as control of the proliferation (p53), adhesion (E-cadherin), differentiation (annexin A2), and survival (p53 and FAK) of cells, and in cell response to hypoxia (HIF-1α).[2,11,12,14-18] Two other processes related with metastases are also studied: apoptosis and angiogenesis. The first is studied through the determination of activated caspase-3 expression, a very reliable indicator of apoptosis,[19] and the second by determining the number of vessels with the CD34 endothelial antigen[20] using a staining technique.

As happened with gene expression, in the study by Roepman et al,[8] we have found that the expression of the proteins analyzed shows a notable correlation between the primary tumour and the metastases, except for the expression of HIF-1α and angiogenesis. In protein expression, there is only 1 prior study that has analyzed several molecular alterations in a high number of cases.[21] That study also found

a similar expression in the metastases and the primary tumour for most (8 out of 10) of the markers analyzed. In addition, the findings coincide with those of our study in the case of the two markers present in both, p53 and E-cadherin. Overall, the 3 studies show that the molecular genetic alterations determining the metastases are already present in the primary tumour.

The protein that revealed the greatest correlation in our study (there was only 1 divergent case) was p53, which is consistent with the fact that its alterations occur early on in carcinogenesis would remain throughout its development. In addition, there was no clear dominance of positive or negative cases, which indicates that the alterations in this protein do not have any role in favouring metastases, as confirmed in previous studies.[15] Nonetheless, the maintenance of the anomalous p53 function in the metastases would allow uncontrolled cell proliferation, as in the primary tumour.[22] With respect to the other proteins that showed a correlation between the primary tumour and the metastases, the expression of E-cadherin and annexin A2 was predominantly negative while that of FAK was positive, as was the apoptotic index. These results are in line with previous studies that have shown a reduction in the expression of E-cadherin in a primary tumour to be related with lymph node metastases,[12,17] as is the diminished expression of annexin A2[18] and the increase in FAK expression.[16] However, the high number of cases with an apoptotic index higher than the mean in this series of cases diverges from other studies showing that the inhibition of apoptosis is related with more aggressive tumours and a higher incidence of metastasis.[23,24] These discrepancies could be explained by the fact that our study only includes patients with metastasis, therefore it would be necessary to analyze the apoptotic index by means of the caspase-3 expression in patients with and without lymph node metastases.

Unlike the previous proteins, the expression of HIF-1α frequently varied between the primary tumour and the metastases. HIF-1α is a protein subject to strong regulation expression due to hypoxia,[25] therefore, in addition to the influence of other genetic and molecular factors that might modify its expression, the different oxygenation conditions in the primary tumour and the metastases might also explain these discrepancies. In connection with this, differences were also found in the degree of vascularization between the primary tumour and the metastases, which would explain the oxygenation differences in both cases and might influence the expression of HIF-1α. In addition, it is likely that the different micro-environment of the cell that exists in the primary tumour and in metastases might influence the phenomena of angiogenesis and hypoxia and so explain the differences found in these aspects in this study.[26]

In conclusion, our study confirms that most of the molecular alterations occurring in lymph node metastases are already present in the primary tumour. This indicates that the capacity to metastasize is already acquired at the early stages of carcinogenesis and is maintained during the tumour's progression. From a practical standpoint, since the molecular alterations favouring metastatic dissemination are present in the primary tumour, their detection would allow the identification of patients with a higher risk of suffering metastasis.

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