Connexin 43 (Cx43) Expression in Laryngeal Squamous Cell Carcinomas: Preliminary Data on Its Possible Prognostic Role

Lidia Puzzo¹ · Rosario Caltabiano¹ · Rosalba Parenti² · Serena Trapasso³ · Eugenia Allegra³

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Abstract The aim of the report is to evaluate the prognostic and predictive role of Connexin 43 (Cx43) expression in laryngeal squamous cell carcinomas. Eighty-seven previously untreated patients submitted to laryngectomy ± neck dissection ± radiotherapy were enrolled in this retrospective study. The original primary tumor slides were reassessed, tumor grade and stage reviewed, and Cx43 immunohistochemical analysis performed: only cytoplasmic membranous staining of Cx43 has been shown. Neither significant correlation has been showed for clinical T ($p = 0.75$) and N ($p = 0.81$), while significant correlation has been found with grading ($p < 0.0001$) and pathological N ($p < 0.0001$). Five year overall survival (OS) of the 87 patients was 54 %; 5 year OS was 59.6 % in Cx43 positive patients and 37.1 % in Cx43 negative patients, but also this difference did not reach statistical significance ($p = 0.058$). Our best findings were: poorly differentiated carcinomas had low or negative Cx43 expression; moderately differentiated tumors without node metastasis and no radiotherapy but with Cx43 expression had a better outcome; moderately differentiated tumors without node metastasis and no radiotherapy but without Cx43 expression had a worse outcome; moderately differentiated tumors with node

metastasis and radiotherapy but without Cx43 expression had a better outcome. Interestingly, in G2 head and neck squamous cell carcinomas with lymph node metastasis at the time of diagnosis, Cx43 aberrant overexpression could identify a subset of patients with poor prognosis, far less responsive to radio/chemotherapy.

Keywords Squamous cell carcinoma · Grading · Staging · Cx43 expression · Prognostic value

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the most common head and neck cancer; despite its frequency, many patients show regional lymph node metastasis or distant metastasis at the time of diagnosis. In the last 30 years, progress in the treatment of head and neck cancer has improved the quality of life of patients through the use of more innovative surgical techniques and multimodal treatments aimed at organ preservation. However, the survival of patients with advanced disease stage has not improved. The main causes of death are represented by the recurrence of loco-regional disease and the appearance of distant metastases not responding to conventional treatments [1]. At present, diagnostic and clinical studies fail to differentiate patients without metastasis or with regional occult metastasis and patients more likely to undergo metastasis from patients with longer overall survival time (OS).

Since the assessment of grading and staging is not enough to evaluate disease progression, it could be useful to identify molecular biomarkers as prognostic and predictive factors. As previously reported in literature, the expression patterns of Gap junction (GJ) proteins seem to

✉ Lidia Puzzo
lipuzzo@unict.it

¹ Department “G.F.Ingrassia”, Section of Anatomic Pathology, University of Catania, Via S. Sofia, 87, 95125 Catania, Italy

² Department of Biomedical and Biotechnological Sciences, Section of Physiology, University of Catania, Via S. Sofia, 64, 95125 Catania, Italy

³ Department of Medical and Surgical Sciences – Section of Otolaryngology, Magna Graecia University of Catanzaro, Viale Europa, Località Germaneto, 88100 Catanzaro, Italy

play a key role in tumor growth and progression [2, 3]. GJs are specialized intercellular channels constituted by about 21 connexins directly connecting the cytoplasm of adjacent cells and involved in metabolic cooperation between cells, growth and developmental regulation; the lack of GJs and Gap Junctional Intercellular Communications (GJIC) could play an important role in tumorigenesis, interacting with cell migration and supporting the ability of invasion and metastasis.

Among 21 connexins, Cx43 is the best known and studied; it is widely expressed in epithelial cells, hematopoietic cells, neurons and astrocytes, cardiac neuronal crest cells, and fibroblasts [4, 5]. Its channel function depends on phosphorylation and dephosphorylation of the C-terminal domain, but recent evidence suggests its potential role as scaffold protein promoting the different assembly of the actin cytoskeleton and microtubules [4, 6]. It seems that the C-terminal domain of Cx43 is responsible either for the tumor suppressor effect or the invasiveness, regulating also the epithelial-mesenchymal transition [7]; therefore the presence of two different activities of Cx43 protein has been proposed, one involving intercellular communication across communicating hemichannels and one acting as scaffold for cytoskeleton associated proteins [4]. Experimental data have shown the role of Cx43 in the control of cell proliferation and apoptosis for its ability to form hemichannels exchanging apoptotic and cell growth factors between extracellular and intracellular matrix, but also its role in promoting tumor progression and metastasis [7]. Recent reports suggest a more complex role of Cx43 in the different stages of tumor progression [4, 7, 8]; Cx43 downregulation has been associated with development of cancer phenotype [2, 9], while aberrant Cx43 upregulation in advanced stages of carcinomas suggests the possibility that cancer cells may communicate with normal host cells across hemichannels, playing a role in migration and growth of metastatic cells. Aberrant Cx43 expression has been detected in advanced stages of prostatic and colonic adenocarcinomas [8, 10], and metastatic cutaneous melanoma [11].

The Authors will evaluate the expression of Connexin 43 (Cx43), as a marker of cellular growth and differentiation, in laryngeal squamous cell carcinomas and its relationship with grading, staging, and overall survival (OS).

Materials and methods

Patients

Previously untreated patients submitted to laryngectomy ± neck dissection at Otolaryngology-Head and Neck Surgery Department of Magna Graecia University,

between 2004 and 2009 were enrolled in this retrospective study. From a total of 106 patients, 19 were subsequently excluded for the poor quality of the reviewed histological samples or because they were lost at follow up; the remaining 87 patients have been included in this study.

The original primary tumor slides were reassessed and tumor grade and stage reviewed by two experienced pathologists (LP and RC) at Section of Anatomic Pathology, “G.F.Ingrassia” Department of Medical, Surgical and Advanced Technology, University of Catania. All patients were followed up every 3 months; the clinical follow-up period was 60 months.

The study has been approved by the Institutional Review Boards; all patients gave their informed consent.

Immunohistochemistry

The surgical specimens were fixed in 10 % buffered formalin, processed routinely, and embedded in paraffin. Four µm thick sections were stained routinely with hematoxylin and eosin and additional sections were cut for immunohistochemical analysis. Antigen retrieval was performed with a heat-induced method in EDTA solution (Target Retrieval Solution, pH high; DakoCytomation, Glostrup, Denmark, 15 min, at 95 °C). All slides were quenched for 5 min in 0.03 % hydrogen peroxide (EnVision + System HRP, DakoCytomation) to block endogenous peroxidase and then incubated in a moist chamber with polyclonal anti-human Cx43 antibody (Sigma; 1:300, 60 min); affinity purified mouse IgG2 was used as a negative control. After washing in TRIS-buffered saline (TBS) pH 7.6, EnVision Flex/HRP (DakoCytomation) was applied for 20 min on the sections. All sections were washed again in TBS; the slides were stained with 3,3'-diaminobenzidine chromogenic substrate (DakoCytomation) for 10 min, counterstained in hematoxylin and mounted in aqueous medium.

Membranous Cx43 expression was evaluated as Her-2 membranous expression in breast carcinomas: no staining or membrane staining that is incomplete and faintly perceptible within less than 10 % of tumor cells (0), membrane staining that is incomplete and faintly perceptible within more than 10 % of tumor cells (1+), membrane staining that is incomplete and moderate within more than 10 % of tumor cells or complete, intense membrane staining within less than 10 % of tumor cells (2+), membrane staining that is complete, intense within more than 10 % of tumor cells (3+).

Statistical analysis

The statistical analysis was performed using MedCalc software (Mariakerke, Belgium) by the Chi square test and Fisher's exact test. A multivariate analysis was performed

using multiple regression tests to determine the independent prognostic factors. All reported p values are two-tailed and $p < 0.05$ was considered significant. Overall survival (OS) was calculated according to the Kaplan–Meier method.

Results

Seventy-seven patients were men and 10 were women; median age was 68 (range 43–86) years. Thirty-eight tumors (43.6 %) had a glottic localization and 49 (56.3 %) had a supra/transglottic localization.

The histological grade was: G1 in 14 cases (16 %), G2 in 45 (51.7 %), and G3 in 28 (32.3 %).

According to the TNM classification (AJCC tumor staging system) primary tumor stage was: T1 in 7 patients (8 %), T2 in 26 (29.8 %), T3 in 37 (42.5 %) and T4 in 17 (19.5 %). Clinical evaluation of regional lymph nodes showed N0 in 28 patients (32.2 %) and N+ in 59 patients (67.8 %).

Sixty-five patients (74.7 %) were treated with selective or modified radical neck dissection and pathological examination of loco regional lymph nodes detected lymph node metastasis in 39 patients, 28 of these underwent post-operative radiation.

Cytoplasmic membranous expression of Cx43 was detected in 52 tumors (59.7 %): 25 of 38 glottic tumors (65.7 %) and 27 of 49 supra/transglottic tumors (55.1 %) were positive, but it was not a significant difference ($p = 0.51$).

A significant correlation has been found between Cx43 expression and histological grade ($p < 0.0001$). All 14 G1 tumors showed Cx43 expression, mainly strong (3+) and moderate (2+) positivity (78.5 %) (Figs. 1, 2); only 3

cases (21.4 %) showed mild Cx43 (1+) expression (Fig. 3); no one was negative.

Thirty-four of 45 (75.5 %) G2 tumors showed Cx43 expression: 29 (85.2 %) showed mild (1+) Cx43 positivity, 5 (14.7 %) showed moderate (2+) expression, no one was strongly positive (3+); the remaining 11 tumors were Cx43 negative.

In this group there were 18 patients with lymph node disease treated with radiotherapy after surgery. Ten out of 18 showed Cx43 positivity and died during follow-up, while 8 were negative and are still living. Twenty-four of 27 patients without lymph node disease showed mild or moderate Cx43 expression and they are still alive.

Twenty-four of 28 (85.7 %) G3 tumors were Cx43 negative, only 4 tumors showed low (1+) Cx43 expression (14.2 %) (Fig. 4).

The multivariate analysis included site, histological grade, T, clinical N, and pathological N and their relationship with Cx43 expression, considering negative Cx43 expression as 0, and 1+, 2+, 3+ Cx43 expression as positive (Table 1).

Evaluating the clinical stage of primary tumor, neither significant correlation has been showed between Cx43 expression and clinical T stage ($p = 0.75$); in fact, Cx43 expression has been detected both in 22 of 33 (66.6 %) T1–T2 stages and in 30 of 54 (55.5 %) T3–T4 stages.

Neither significant correlation has been found between Cx43 expression of primary tumor and clinical lymph node status ($p = 0.81$): 23 of 59 (39 %) patients with clinical metastatic lymph nodes were Cx43 negative, but also 12 of 28 (42.8 %) patients without clinical lymph node metastasis were Cx43 negative.

Instead, a significant statistical correlation has been found between Cx43 expression of primary tumor and

Fig. 1 High Cx43 expression (3+) in G1 squamous cell carcinoma (IHC $\times 10$, inset $\times 40$)

Fig. 2 Moderate Cx43 expression (2+) in G2 squamous cell carcinoma (IHC $\times 10$, inset $\times 40$)

Fig. 3 Low Cx43 expression (1+) in G2 squamous cell carcinoma (IHC $\times 10$, inset $\times 40$)

Fig. 4 Negative Cx43 expression in G3 squamous cell carcinoma (IHC $\times 20$)

pathological lymph node status ($p < 0.0001$): 21 of 26 (80.7 %) patients without metastatic lymph nodes showed Cx43 expression in the primary tumors, while only 15 of 39 (38.4 %) patients with metastatic lymph nodes showed low Cx43 expression.

The 5 year overall survival (OS) of the 87 patients was 54 % (47/87); 5 year OS was 59.6 % (31/52) in Cx43 positive patients and 37.1 % (13/35) in Cx43 negative patients; nevertheless, this difference did not reach statistical significance ($p = 0.058$) (Fig. 5).

Discussion and conclusions

Advanced laryngeal squamous cell carcinomas are usually characterized by regional lymph node metastasis at the time of diagnosis and higher incidence of recurrences during the follow up; both represent adverse prognostic

Table 1 Clinical and pathological data and Cx43 expression

Clinical data	N.87	Cx43 expression	
		Negative	Positive
Localization			
Glottic	38	14	25
Supra/transglottic	49	21	27
			$p = 0.51$
Histological grade			
G1	14	0	14
G2	45	11	34
G3	28	24	4
			$*p < 0.0001$
T classification			
T1	7	2	5
T2	26	9	17
T3	37	16	21
T4	17	8	9
			$*p = 0.75$
cN status			
N0	28	12	16
N+	59	23	36
			$p = 0.81$
pN status			
N0	26	5	21
N+	39	24	15
			$p < 0.0001$

* Chi square test

Fig. 5 5-Year overall survival in negative and positive Cx43 patients

factors in clinical outcome and decreased overall survival by about 50 %. Although clinical management of patients (palpation, computed tomography, magnetic resonance of head and neck district), clinical staging could not be useful to identify lymph node metastasis and 7–37 % of patients

with N0 clinical stage could hide occult metastatic lymph nodes.

Since the assessment of grading and staging is not enough to evaluate disease progression, it could be useful to identify molecular biomarkers as prognostic and predictive factors [12–15].

To improve the diagnostic accuracy, the authors propose to evaluate retrospectively the expression of Cx43 in 87 laryngeal squamous cell carcinomas with known clinical data, grade and stage; the choice to evaluate Cx43 expression is derived from the most recent knowledge about the role of GJs (Connexins) in the process of inhibiting or promoting migration and invasion of cancer cells [16].

As already known, the passage of small proteic molecules across GJs permits the exchanges between cytosol and extracellular space; moreover, the hemichannels of adjacent cells can directly connect their cytosol and transfer molecular signals from cell to cell, as bystander effect [3, 7]. GJs play a physiological role either as regulators of homeostasis or as vehicles of apoptotic signaling regulators in tissue development and differentiation.

Furthermore, all of the studies have revealed that the different and opposite functions of Cx43 depend on its relationship with other known molecular patterns regulating cellular proliferation, metabolism, migration, and survival; these divergent mechanisms have been involved in a different therapeutic approach. *In vitro* data have shown that, in case of Cx43 downregulation, the GJ restoration by target therapy was able to increase the penetrance of chemotherapeutic drugs, influencing the choice of adjuvant cancer therapy; in case of Cx43 upregulation of advanced stage tumors, connexin inhibitors may control growth and invasiveness of metastatic cells [5, 16].

Lastly, a potential prognostic role of Connexins has been proposed in several cancers [7, 17, 18, 22]: in colorectal adenocarcinomas, the loss of Cx43 expression in cancer cells has been linked with poor clinical outcome in I-II stages, suggesting a Cx43 prognostic role to identify a subgroup of high-risk patients, not submitted to chemo/radioadjuvant therapy, for the standard guidelines [7]. Further studies could be performed to demonstrate the opportunity to submit to chemo/radioadjuvant therapy those patients with colorectal adenocarcinomas in I-II stages without Cx43 expression.

Very few studies have been reported about Cx43 expression in head and neck squamous cell carcinomas, mainly in laryngeal squamous cell carcinoma [19–21]: a previous report about expression of 26, 30, 32 and 43 Connexins in normal mucosa, hyperkeratosis and laryngeal carcinoma had shown their irregular distribution and the need of further investigations about their potential prognostic role; in 2006, Frank et al. had detected *in vitro* Cx26

and Cx43 expression in head and neck normal and cancer cells, suggesting the potential role of Cx43 expression in therapeutic response of normal cells near cancer cells, for bystander effect.

In our paper, high or moderate Cx43 expression has been detected in overall well differentiated squamous cell carcinomas (G1), while low or negative Cx43 expression has been found in poorly differentiated carcinomas (G3): as expected, these data confirmed the relationship between low and high grade carcinomas and Cx43 expression.

Cx43 expression in moderately differentiated carcinomas (G2) had shown more interesting assessment; G2 carcinomas without lymph node metastasis (N-) showed low or moderate (1+ or 2+) Cx43 expression and were living during the follow up. G2 carcinomas with metastatic lymph nodes at the time of diagnosis (N+) showed variable Cx43 expression and detected two subsets of patients with potentially different prognosis: the patients with low/moderate Cx43 expression, submitted to post surgical radiotherapy, died during the follow up, while the patients without Cx43 expression, submitted to post surgical radiotherapy, are still alive.

According to the previous literature data about Cx43 expression in colorectal adenocarcinomas, also in head and neck squamous cell carcinomas with lymph node metastasis at the time of diagnosis, Cx43 aberrant overexpression could identify patients with poor prognosis, far less responsive to radio/chemotherapy, while Cx43 downregulation could identify patients with better behavior, more responsive to radio/chemotherapy.

Further studies on enrolled patients could be performed to confirm our preliminary results and potentially utilize Cx43 expression as a useful prognostic and predictive biomarker of laryngeal squamous cell carcinoma behavior.

Compliance with Ethical Standards

Conflict of interest None declared.

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