Türk Otolarengoloji Arşivi
Turkish Archives of Otolaryngology

Received / Geliş tarihi: February / Şubat 15, 2010
Accepted / Kabul tarihi: March / Mart 8, 2010
Published online / Online yayın tarihi: March / Mart 23, 2010
doi: 10.2399/tao.10.009

ARAŞTIRMA / RESEARCH ARTICLE

Is there any neuroendocrine differentiation in squamous cell carcinoma of larynx?

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Larenks yassı hücreli kanserinde nöroendokrin farklılaşma bulunabilir mi?

Amaç: Larenks kanserinde nöroendokin (NE) marker ekspresyonunu araştırarak prognostik ilişkilerini tespit etmek.

Yöntem: Prospektif özellikteki bu çalışmaya 30 hasta dahil edildi. Tümörler diferansiyasyon miktarına göre derecelendirildi. Immünohistokimya (İHK) ile nöron spesifik enolaz (NSE), sinaptofizin (SYNP) ve kromogranin A (ChrA) spesifik antikorları kullanılarak immün reaktivite araştırıldı. Sonuçlar boyanan hücre yüzdesine göre 5 seviyede derecelendirildi.

Bulgular: Bu çalışmada ameliyat sonrası 5 ila 38 ay takip edilen larenks yassı hücreli karsinom (YHK) ve varyantları bulunan 30 hasta mevcuttur. Seride 13 total, 17 parsiyel larenjektomi bulunmaktaydı. Grade II YHK en sık görülen tümördü (%63.3). İHK değerlendirmesinde yalnızca adenoskuamöz kanserli bir olguda seviye 1 ChrA pozitifliği izlendi (<%5). SYNP ile 8 olgu (%27.6) seviye 1, 2 olgu (%6.9) seviye 2 pozitiflik gösterdi. Bu pozitiflik ileri evrede erken evreye göre kısmen daha sık izlendi (%24.1'e karşılık %10.3, p=0.433). NSE ile 14 olguda (%48.3) yer yer 5. seviyeye varan pozitiflik görüldü.

Sonuç: Bu ön çalışmada, non-NE larinks tümörlerinde NSE ve SYNP markerları ile NE farklılaşma varlığı dikkati çekmektedir. Sınırlı sayıdaki hasta ile İHK sonuçlarıyla hastalığın biyolojik davranışı ve sağkalım arasında bağıntı kurmak mümkün olmamıştır. Ancak hasta sayısı artırıldığında ve daha sensitif markerlar kullanıldığında, NE markerların larenks kanserinde biyolojik davranışı anlamaya yönelik yeni bir gereç olabileceğini düşünmekteyiz.

Anahtar Sözcükler: Larinks kanseri, prognostik belirteçler, farklılaşma belirteçleri, nöron spesifik enolaz, sinaptofizin, kromogranin A.

Türk Otolarengoloji Arşivi, 2010; 48(1): 28-36

Abstract

Objectives: To investigate the expressions of distinct neuroendocrine (NE) markers in larynx cancer to find out possible prognostic implications.

Methods: Thirty patients were evaluated in this prospective study. Tumors were graded according to the degree of differentiation. Immunohistochemistry (IHC) was performed using antibodies against neuron specific enolase (NSE), synaptophysin (SYNP), and chromogranin A (ChrA). Staining reactivity was graded semi-quantitatively as percent of stained cells into 5 levels.

Results: The study consisted of 30 patients with squamous cell carcinoma (SCC) and its variants that were followed up for 5 to 38 months. Specimens included 13 total and 17 partial laryngectomies. Grade 2 SCC was the most prevalent type (63.3 %). On IHC evaluation, only one tumor with adenosquamous carcinoma features showed cytoplasmic ChrA (<5%). Ten tumors showed SYNP positivity (as rate 1 in 8 cases [27.6%] and rate 2 in 2 cases [6.9%]). SYNP expression was slightly more common in advanced stage than early stage (24.1% vs 10.3%, p= 0.433). With NSE, 14 tumors showed (48.3 %) positive expression where it reached rate 5 (76-100%) staining in some of them.

Conclusion: In this preliminary study, we were able to describe NE differentiation with NSE and SYNP positivity in various types of non-NE laryngeal tumors. Though we could not put forth significant correlation of our results with survival and biological behavior of the disease, we believe that further investigation in a larger series is needed to define the role of NE differentiation as a biomarker of biological behavior in some laryngeal cancers.

Key Words: Laryngeal cancers, prognosis, differentiation markers, neuron-specific enolase, synaptophysin, chromogranin A.

Turk Arch Otolaryngol, 2010; 48(1): 28-36

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Introduction

According to United States National Cancer Database over a 10 year period, larynx is the most common site of head and neck cancer, accounting more than 20% of the cases.[1] About 9.500 new larynx cancer (LC) patients were seen in 2006 and about 3.700 deaths have occurred due to LC in United States.[2] A century ago, only treatment option for LC was total laryngectomy, which is a modality that significantly decreases the quality of life. Following increase in knowledge in tumor biology, a number of organ preservation treatment options now exist, and significant effort is still going on to preserve laryngeal functions and improve survival in larger amounts of patients in such a frequent disease.

Prognosis of the LC is known to be related with certain clinical indicators as laryngeal localization and stage of the disease and histological parameters like the differentiation of the tumor. However these clinical features can be unreliable predictors for individual patients and several features can only be investigated after surgical resection. Therefore there is still need for further prognostic indicators at the time of diagnosis for predicting treatment response.[3] So far, most of the research has been focused on expression of proteins involved in cell cycle regulation, signal transduction, apoptosis, cell adhesion or cell–extracellular matrix interaction and significant progress has been accomplished, but question about why some tumors behave more aggressively and metastasize more frequently but others do not still remains.[3]

Non-small-cell lung cancers are also epithelium derived smoking related neoplasms of the respiratory tract.[4] Despite non-neuroendocrine in origin, 10-20% non-small-cell lung cancer were shown to express neuroendocrine (NE) elements by means of immune electron microscopy and immunohistochemistry (IHC) and their existence has been shown to effect prognosis.[4] Additionally, in non-small–cell carcinomas of the lung, cervix, colon, and prostate, the presence of NE elements were also found to be associated with a better[4] or worse[5-7] prognosis in some studies, or had no prognostic effect in other studies.[8] Therefore we planned to conduct a preliminary prospective study to investigate presence of NE differentiation and their prognostic meanings in non-neuroendocrine larynx cancers with IHC.

Materials and Methods

Thirty LCs diagnosed and operated in the authors-affiliated institution were evaluated in this prospective study. The study consisted of patients with squamous cell carcinoma (SCC) and its variants that were followed up for 5 to 38 months (mean 20.5 months), prospectively. None of the patients had been treated before surgery. The patients were staged according to the TNM system (6th Edition; UICC, International Union against Cancer). The recorded clinicopathologic features of the tumors were patient age, gender, tumor site, tumor grade, type of operation, clinical and pathological stages, and clinical follow-up data (Table 1).

Histopathological evaluation

All specimens; laryngectomy, and neck dissection, were fixed and processed routinely. Hematoxylin and eosin (HE) stained slides were evaluated for diagnosis, growth (i.e. ulcerous, papillary or keratotic), and infiltration (i.e. expanding, or infiltrating) patterns, and surgical margins. Neck dissection specimens were handled with routine evaluation protocol. Tumors were graded according to the degree of differentiation; cellular pleomorphism, nuclear properties and mitotic index as follows: well differentiated (grade 1; G1), moderately differentiated (grade 2; G2)

Table 1. Demographic data of patients, localization, TNM classification and stage of the tumors and treatment results are shown (Except subject 7, all subjects were male).

Case	Age	Location	T	N	M	Stage	Surgery	Neck dissection	Adjuvant RT	Result
1.	57	G	1	0	0	1	VPL	L- FND	NO	NED
2.	42	SG	2	0	0	2	SHL	R- FND	NO	NED
3.	59	G	2	0	0	1	LFC	NONE	YES	NED
4.	61	TG	4	2B	0	4A	TL	R- MRND	YES	NED
5.	54	TG	2	2C	0	4A	SHL	R- FND	YES	Dead*
6.	63	G	1	0	0	1	FLL	NONE	NO	NED
7.	69	TG	2	0	0	2	3\4 L	L- FND	NO	NED
8.	47	G	1	0	0	1	LFC	NONE	YES	NED
9.	58	TG	4	1	0	4A	TL	R- FND	YES	NED
10.	62	TG	3	2B	0	4A	TL	L- RND	YES	NED
11.	60	TG	3	1	0	3	TL	BIL FND	YES	NED
12.	43	TG	3	0	0	3	TL	NONE	YES	NED
13.	60	SG	2	1	0	3	SHL	L- MRND, R- FND	YES	Dead**
14.	62	SG	2	0	0	2	SHL	R- FND	NO	NED
15.	53	G	1A	0	0	1	LFC	NONE	YES	NED
16.	64	G	3	0	0	3	TL	L- FND	YES	NED
17.	70	G	2	0	0	2	VPL	NONE	NO	NED
18.	59	TG	2	0	0	2	VPL	NONE	NO	NED
19.	65	G	1	0	0	1	LFC	NONE	NO	NED
20.	60	G	2	0	0	2	VPL	NONE	NO	NED
21.	58	TG	3	0	0	3	TL	NONE	YES	NED
22.	63	TG	4	2C	0	4A	TL+Partial pharyngectomy	R- RND	YES	NED
23.	64	SG	2	2C	0	4A	TL	R- FND	YES	NED
24.	47	SG	2	2	0	4A	3\4 L	R- FND	YES	NED
25.	43	TG	3	0	0	3	TL	BIL FND	NO	NED
26.	50	G	2	1	0	3	TL	R- FND	NO	NED
27.	37	SG	1	0	0	1	SHL	BIL FND	NO	NED
28.	53	TG	3	0	0	3	TL	L- FND	YES	NED
29.	41	TG	2	0	0	2	SCPL	NONE	NO	NED
30.	53	SG	4	0	0	4A	TL	BIL FND	NO	NED

:** Disease specific death; *:** Disease non-related death, **NED:** No evidence of disease; **G:** Glottic; **S:** Supraglottic; **T:** Transglottic; **L:** Left; **R:** Right; **BIL:** Bilateral; **VPL:** Vertical partial larygectomy; **SHL:** Supraglottic horizontal laryngectomy; **LFC:** Cordectomy with laryngofissure; **3\4 L:** 3\4 laryngectomy; **SCPL:** Supracricoid partial laryngectomy; **TL:** Total laryngectomy; **FND:** Functional neck dissection; (**M)RND:** (Modified) Radical neck dissection

and poorly differentiated (grade 3; G3).[9] Mucosa adjacent to tumor was evaluated for the type of mucosa; stratified, pseudostratified or immature stratified epithelium, and for the mucosal changes according to the Ljubljana Classification.[10] Tumors were grouped by stage as early stage (ES; stage I and II), or advanced stage (AS; stage III and IV).

Immunohistochemistry

Immunohistochemistry was performed using antibodies against neuron specific enolase (NSE), synaptophysin (SYNP), and chromogranin A (ChrA). One paraffin-embedded tissue per case from a representative tumor area with adjacent non-tumoral mucosa was used for IHC. The expression

of markers was evaluated on 4 μm-thick sections mounted on to positively charged slides, and performed in an automatic immunohistochemistry staining machine (BenchMark XT Staining Module, Ventana Medical Systems) by using streptavidin-biotin peroxidase method.[11] After dewaxing, slides were incubated with primary antibodies; NSE (Neomarkers; E27, citrate, 1:25), SYNP (Neomarkers; Ab-4, citrate, 1:40), and ChrA (Dako; DAK-A3, citrate, 1:50). Counter-stain was performed with Mayer's hematoxylin. Positive controls were stained in parallel. The evaluation of IHC was based on intensity and distribution of the markers. Staining distribution of the markers was rated as: rate 1 (0-5%), rate 2 (6-25%), rate 3 (26-50%), rate 4 (51-75%), and rate 5 (76-100%).

Statistical analysis

Staining distributions and prognostic indicators were compared with Chi-square statistics and correlated with Spearman's non-parametric correlation coefficient tests using computer software (Statistical Package for Social Sciences (SPSS), 11th version, SPSS Inc. Chicago, Illinois).

Results

The age of the patients; 29 male, one female, was ranged from 37 to 70 (averagely 56 yrs). Ten tumors were localized in glottis, 7 in supraglottis, and 13 were transglottic. Laryngectomy specimens included 13 total laryngectomy (TL), and 17 partial laryngectomy (PL). Fourteen patients (46.7%) were at ES, and 16 (53.3%) were at AS (Table 1).

The number of tumors with G2 features was highest (19 / 63.3%) whereas G3 tumors were only 5 (16.7 %). G1 tumors included 3 SCC (10%) and 3 verrucous carcinoma (VC) (10 %). Tumors with G3 features included 1 SCC (3.33%), 2 sarcomatoid carcinoma (SC) (6.67 %), 1 basaloid SCC (BSCC) (3.33%), and 1 adenoquamous carcinoma (ASC) (3.33%).

Clinically N+ 10 cases (33.3%) had neck dissection and 8 of them (26.6%) were confirmed to be metastatic to lymph node, histologically (Tables 1 and 2). In the present study, most of the tumors (63.3 %), as well as metastases (6 out of 8), were moderately differentiated SCC. One metastasis revealed a well differentiated SCC histology, while another one was BSCC. Although, tumor differentiation and cervical lymph node metastases was not significantly correlated due to the number of tumors studied (p=0.58), the correlation between primary tumor location and neck metastases reached statistical significance (p=0.05). In 5 metastases the primary tumors were transglottic, while in 3 metastases the tumors were in supraglottis.

Five tumors showed positive surgical margin(s) (Table 2) and were subjected to adjuvant radiotherapy (RT). Subglottic invasion was found in 6 tumors treated with TL of whom 2 also demonstrated surgical margin invasion and treated with RT. Seven patients required extended TL due to extralaryngeal tumor spread of whom 5 were given RT, and 2 were chosen for wait and see, as they had VC and the tumors were believed to be removed totally. In 8 of 19 neck dissection specimens, metastases with extracapsular spread were demonstrated. Those patients were treated with adjuvant RT and one of them developed neck recurrence and died of the disease in 29th month after surgery. Other recurrent tumor was seen after open cordectomy and treated with RT. He received chemotherapy for colon cancer on the postoperative 26th month and had no evidence of local or distant recurrence due to LC. One patient was lost on postoperative 20th month due to atherosclerotic heart disease, without evidence of recurrence of LC (Table 1).

Table 2. Histopathological findings and immunohistochemistry results.

Case	Pathology/Grade	Surgical border inv.	Cartilage inv.	Subglottic inv.	Extralaryngeal inv.	MET/ LAP	NSE	SYNP	ChrA
1	2	-	-	-	-	0	0	0	0
2	2	+	-	-	-	0	0	0	0
3	1	-	-	-	-	0	3	0	0
4	2	-	+	+	+	1 (R)	1	0	0
5	2	-	-	-	-	6 (R),1(L)	0	0	0
6	SC	-	-	-	-	0	0	0	0
7	SC	-	-	-	-	0	0	0	0
8	2	+	-	-	-	0	0	0	0
9	2	-	-	-	+	2 (R)	0	0	0
10	2	-	-	+	-	0	0	1	0
11	2	-	-	-	-	3 (R),1 (L)	0	0	0
12	ASC	-	-	-	-	0	0	1	1
13	2	-	-	-	-	2 (L)	0	0	0
14	2	-	-	-	-	0	3	1	0
15	1	+	-	-	-	0	0	0	0
16	2	+	+	+	+	0	0	0	0
17	2	-	-	-	-	0	0	1	0
18	2	-	-	-	-	0	4	1	0
19	2	-	-	-	-	0	N/A	N/A	0
20	VC	-	-	-	-	0	2	0	0
21	3	+	+	+	+	0	5	1	0
22	1	-	+	-	+	5 (R)	3	2	0
23	2	-	-	-	-	1 (R)	5	1	0
24	BSSC	+	-	-	-	1 (R)	5	2	0
25	VC	-	+	+	+	0	1	1	0
26	2	-	-	+	-	0	2	0	0
27	2	-	-	-	-	0	0	0	0
28	2	-	-	-	-	0	5	0	0
29	2	-	-	-	-	0	2	0	0
30	VC	-	+	-	+	0	3	0	0

L: Left; **R:** Right; **SCC:** Squamous cell carcinoma; **MET:** Metastases; **LAP:** Metastatic lymph node number; **SC:** Sarcomatoid carcinoma; **VC:** Verrucous carcinoma; **ASC:** Adenosquamous carcinoma; **NSE:** Neuron specific enolase; **SYNP:** Synaptophysin; **ChrA:** Chromogrannin A

Immunohistochemical features

Cytoplasmic expression of the markers was shown in Table 2. Only ASC (Case 12) showed ChrA expression in some cells (0-5%) of which were also positive for SYNP in the same extent (Figure 1A, B). IHC and consecutive HE stained slides demonstrated that immunoreactive cells were glandular structures entrapped by the tumor invasion. ChrA was positive in neither non-tumoral mucosa, nor in other tumor types. Of 29 tumors searched for SYNP expression, 8 (27.6%) showed rate 1 SYNP positivity and 2 (6.9%) were positive at a rate of 1. The rate of expression was slightly more common in the tumors with AS than ES (24.1% vs 10.3%, p=0.433). SYNP showed non-specific single cell expression around keratin pearls (Figure 1C), and in the islands of squamous epithelium. Closer look to these islands were demonstrated SYNP positive kera-

tinized epithelium surrounded by foreign body type inflammatory reaction. No SYNP expression was detected in stratified, pseudostratified or immature stratified laryngeal epithelium.

Only 2 tumors showed rate 2 SYNP distribution. First was BSCC (Case 24), demonstrated SYNP positive cells at the periphery of tumor islands (Figure 1D) where NE differentiation could be expected.[12] Other tumor was a AS transglottic cancer with G1 SCC features showing SYNP expression in non-keratinized poorly differentiated tumor cells.

Of the 29 tumors stained for NSE, 14 (48.3 %) showed immunoreactivity which was strong and diffuse in some of them including a BSCC (Figure 2A). The expression was mostly in the keratinized cell layer in G1 tumor islands (Figure 2B), but it extended through the deeper layers in some cases. In the invasive tumor islands, NSE came into sight in central better differentiated areas and extended to the less differentiated cells (Figure 2C). No NSE expression was detected in stratified, pseudostratified or immature stratified laryngeal epithelium. The case with basaloid SCC had also showed very wide NSE expression.

Adjacent non-tumoral mucosa showed no staining with the markers used. Therefore the NE differentiation defined by Chr A, NSE and SYNP immunoreactivity in tumor samples belonged to neoplastic process. However no significant correlation could be observed between survival, clinical findings and IHC staining results and these results were failed to be used for prognostication in this small series.

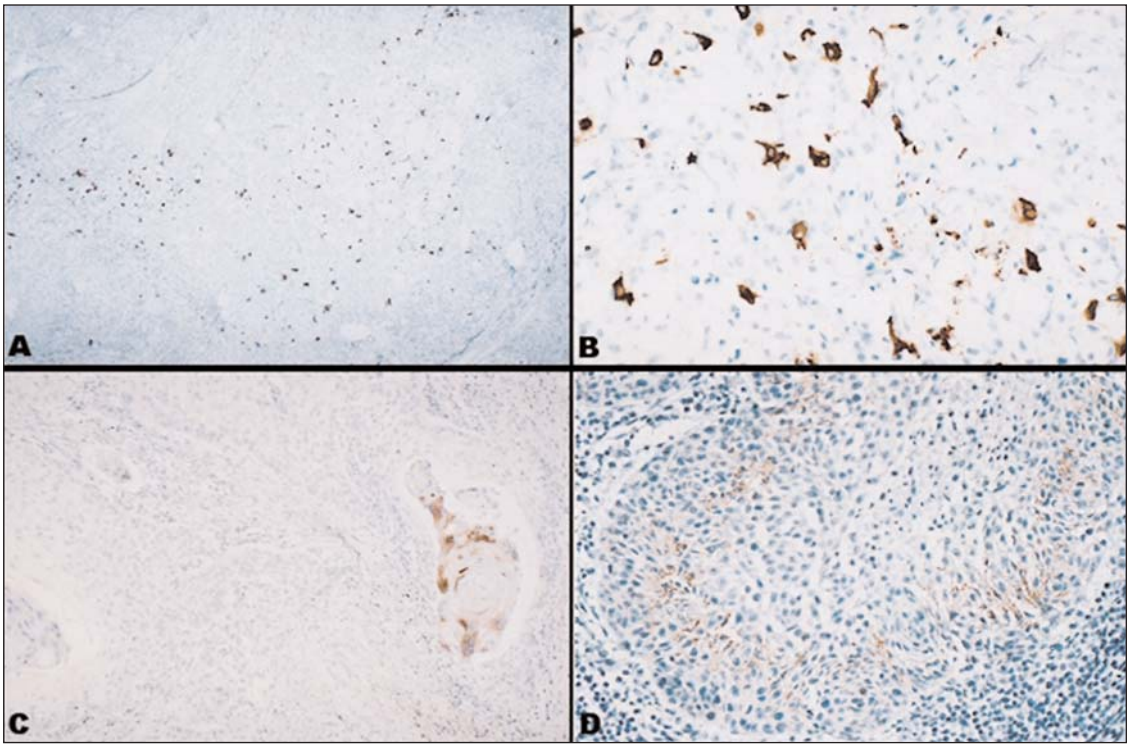

Figure 1. Scattered chromogranin A positive cells in adenosquamous carcinoma (**A**, peroxidase, x2.5) which belong to glandular structures entrapped by the tumor invasion (**B**, peroxidase, x50). Non-specific and specific synaptophysin expression in single cells around keratin pearls (**C**, peroxidase, x25), and in poorly differentiated cells (**D**, peroxidase, x50). [Color figure can be viewed in the online issue, which is available at www.turkarchotolaryngol.org]

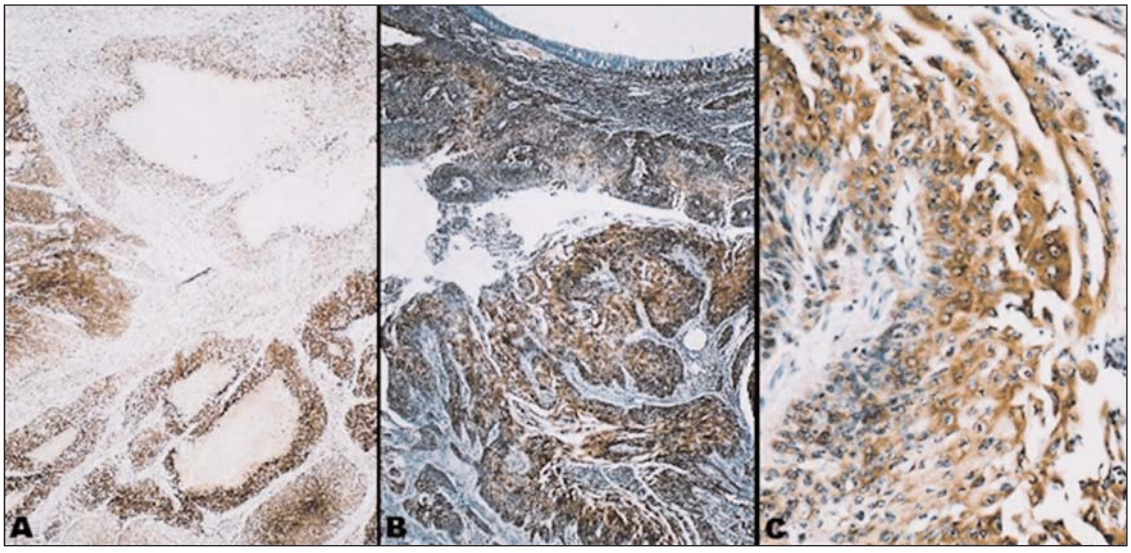

Figure 2. Neuron specific enolase expression in basaloid squamous cell carcinoma (**A**), and in squamous cell carcinoma (**B** and **C**) (peroxidase, x10, x10, x25). [Color figure can be viewed in the online issue, which is available at www.turkarchotolaryngol.org]

Discussion

To the best of our knowledge, this is the first study evaluating the presence of NE differentiation in larynx cancer that encompassed cases with SCC of all grades, and SCC variants; ASC, VC, SC and BSCC. Larynx is covered mainly by non-keratinized stratified squamous epithelium and more than 90% of LCs are SCC. However, as many other foregut derived organ, larynx mucosa includes the elements of diffuse neuroendocrine system (DNES), either dispersed or cluttered as "neuroendocrine corpuscles".[13,14] These are multipotent cells and may also go through malignant transformation which results in known neuroendocrine tumors of larynx that are carcinoid tumor, atypical carcinoid tumor and small cell carcinoma.[13] As a group, these are aggressive tumors with high local and distant metastases rate and unfavorable prognosis. Since these tumors are originated from multipotent progenitor cells that may have potential of epithelial and/or neuroendocrine differentiation, they may show both SCC and NE elements throughout the tumor.[15,16] There may be NE differentiation in some lung, bronchial and cervix SCCs which was shown to change the overall prognosis and biologic behavior of the disease.[4-7] Therefore, we investigated NE differentiation in non-NE tumors of larynx and its correlation with known prognostic factors. To evaluate NE differentiation ChrA, SYNP and NSE immunoreactivity of the tumor cells were searched.

Chromogranin A, an acidic glycoprotein, normally found in the matrix of dense-core NE granules is synthesized in most cells that belong to DNES.[17] Thus, ChrA is a very sensitive and specific NE marker.[17] Although, ASC in the present series demonstrated ChrA positivity, it was not in the tumor cells; instead it belonged to entrapped glandular tissue and did not represent a NE differentiation of ASC. In a similar study, Fresvig et al. investigated NE differentiation in 29 bronchial SCC using ChrA immunoreactivity.[18] With conventional immuneperoxidase staining they found no ChrA expression but using a more sensitive IHC method; the thyramide signal amplification, they

were able to show ChrA expression in 10 tumors.[18] They concluded that when a more sensitive IHC technique is used, a fairly large fraction of bronchial SCCs can be shown to display a NE phenotype in the most undifferentiated parts of the tumor. Additionally, they also investigated ChrA expression in normal bronchial mucosa but they found no expression with conventional IHC or using thyramide signal amplification, and they claimed that NE differentiation develops during metaplasia-malignant transformation process.[18] This shows that, to demonstrate a NE differentiation with ChrA, if any, there is need to use more sensitive IHC methods.[19]

In this study, SYNP and NSE revealed fairly disseminated expression compared to ChrA. SYNP is an integral glycoprotein over presynaptic vesicle membrane and expressed both in neural and epithelial type of NE tumors.[20] NSE is an izoenzyme of the glycolitic enzyme enolase that is specific for NE cells.[13,14,19] NSE is accepted to be NE marker of less certainty and it is important not to rely on NSE expression alone, instead of combined NSE with specific markers of NE differentiation.[19] SYNP expression in our series was mostly attributed to inflammatory reaction around keratin pearl areas. Although, distribution of NSE expression was wider than SYNP, including less differentiated SCCs and BSCC, it did not show any significant correlation with clinicopathologic features.

One of the problems in evaluating NE differentiation in a non-NE tumor is the lack of a definition or "gold standard" of NE differentiation.[21] The definition closest to the original description of a NE cell, with mixed neural and endocrine morphology, requires detection of dense core vesicles in the cytoplasm.[21] For diagnostic purposes, IHC is a cost-efficient way to show NE elements. However during malignant transformation cells may undergo some morphological changes that may obscure or change marker expression. Ultrastructural detection of cytoplasmic NE vesicles in tumors with immune electron microscopy would be the most reliable finding of NE differentiation.[21] However this is an expensive and complex method that would be nearly impossible to perform in routine basis. Thus we believe that our results should be checked using more sensitive detection methods as thyramide signal amplification or catalyzed signal amplification in larger series of patients with longer follow up. Immune electron microscopy can be planned further to explain the results in selected samples.

In summary, in this preliminary prospective study, we were able to describe NE differentiation in various types of non-NE LCs by NSE and SYNP expressions. We could not put forth significant correlation of our results with current prognostic factors, biological behavior of the disease and survival. However, we believe that, further investigation of NE differentiation in larger series, by using more sensitive IHC methods, may mediate to demonstrate hidden NE component of these tumors and may serve as a promising biomarker of biological behavior in LC.

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Conflict of interest statement:
No conflicts declared.

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