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Squamous cell carcinoma in Barrett's esophagus: field effect versus metastasis

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SUMMARY

Barrett's esophagus (BE) is a premalignant condition with an increased risk of developing esophageal adenocarcinoma (EAC). Risk factors for EAC overlap with those for esophageal squamous cell carcinoma (ESCC), but ESCC is surprisingly rare in BE. We report two cases of ESCC directly surrounded by BE. Both patients had a previous medical history of cancers, i.e., head and neck squamous cell carcinomas, and were using alcohol and smoking tobacco. Using immunohistochemistry for p63, CK5, CK7, and CDX2, it was confirmed that these carcinomas were pure squamous cell carcinomas, and not EACs or esophageal adenosquamous carcinomas arising from BE. Using *TP53* mutation and loss of heterozygosity analysis, we established that the ESCCs in BE were not metastases of the previously diagnosed head and neck squamous cell carcinomas but *de novo* primary ESCCs. This study shows the strength of molecular analysis as an adjunct to the histopathologic diagnosis for distinguishing between metastases of prior cancers and primary cancers. Furthermore, these cases imply that presence of BE is not protective with regards to developing ESCC in the lower one third of the esophagus. We suggest that their ESCCs arose from islets of squamous epithelium in BE.

Keywords

adenocarcinoma; Barrett's esophagus; squamous cell carcinoma

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition of the esophagus, which is frequently diagnosed in Caucasian men.¹ It is a well-established disorder proximal to the gastroesophageal junction, characterized by the presence of intestinal metaplasia (IM) and goblet cells in biopsy samples.²

Esophageal cancer (EC) is the seventh most common cause of cancer-related mortality in the USA. Despite the availability of advanced endoscopic, surgical, and medical treatment modalities, the prognosis of patients with EC is still poor with a 5-year survival rate of

15%.³ Although the incidence of EC is relatively low, over the past decades, it is increasingly being diagnosed in Western countries.⁴

Two major types of cancers that can be recognized in the esophagus are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), with the latter accounting for approximately two thirds of cases in the Western world.³ It has been established that most, if not all, EAC cases arise in association with BE. The risk of EAC in BE is approximately 0.3–2% per annum.² Although EAC is strongly associated with BE, less than 10% of patients presenting with EAC are known with a prior diagnosis of BE.⁵ Risk factors for developing EAC in BE include longer duration and higher frequency of gastroesophageal reflux disease, Caucasian and Hispanic race, male sex, advancing age, use of tobacco, and obesity.² Risk factors for ESCC overlap with those of EAC, but surprisingly, ESCC is rarely encountered in patients with BE.

Herein, we report two cases of ESCC arising in BE. In order to confirm that these ECs were indeed ESCCs, and not EACs, or esophageal adenosquamous carcinomas, tumor samples were stained for p63, cytokeratins 5 (CK5) and 7 (CK7), and CDX2. As both patients had been treated for head and neck squamous cell carcinoma (HNSCC) in the past, we furthermore investigated whether these ESCCs were primary cancers that arose in a background of BE or metastases from prior HNSCCs by performing *TP53* mutation analysis and loss of heterozygosity (LOH) analysis.

THE CASES

Case A

A 65-year-old white man presented in 2010 with retrosternal pain and progressive dysphagia for solid foods. There was no weight loss. The previous medical history included a moderately differentiated HNSCC in the oral cavity (T2N0M0), which had been treated with resection and adjuvant radiotherapy in 2007, and prostate cancer, which was resected 2 years before. The patient had quit smoking in 2007 and used moderate amounts of alcohol. Physical examination showed a body mass index of 23.9 but was otherwise unremarkable. Upper endoscopy was performed, which revealed a tumor with an ulcerative surface with in a short BE segment (Fig. 1). Endoscopic ultrasound showed enlarged lymph nodes surrounding the esophagus. Histological examination of endoscopic biopsies revealed a moderately differentiated squamous cell carcinoma (SCC) immediately adjacent to BE mucosa. The tumor was a pure ESCC; there were no adenocarcinoma components found. The adjacent squamous mucosa showed high-grade dysplasia. The ESCC was staged as T3N1M0. The patient underwent esophageal resection.

Case B

A 73-year-old white man underwent upper endoscopy for surveillance of BE in 2009. At the time of admission, the patient had no symptoms. His previous medical history included HNSCC in the trigonum retromolare (T3N0M0), in the oropharynx (T1N0M0), and on the tongue (T1N0N0). All three lesions were resected in 2000. He again had an HNSCC of the base of the tongue in 2006. His previous medical history revealed cardiovascular disease and hepatic cirrhosis secondary to severe alcohol use. In 2008, he quit smoking. Physical examination was unremarkable. Upper endoscopy showed a nodular lesion in a BE segment (Fig. 2), which was histologically confirmed to be a poorly differentiated ESCC surrounded by areas of IM. The ESCC had arisen from a squamous high-grade epithelial lesion. No evidence for an adenocarcinoma component was present. The ESCC extended into the muscularis mucosae but did not involve the submucosa and was staged as T1aN0M0. An endoscopic mucosal resection was performed, followed by ablative treatment of the remaining BE segment. During the latest follow-up visit, 2.5 years later, no signs of

recurrent ESCC have been found. In 2009, the patient was also diagnosed with colorectal polyps (one with high-grade dysplasia) and adenocarcinoma of the ascending colon for which a subtotal colectomy was performed. In addition, laser treatment of an HNSCC of the palatum durum was performed in the same year.

MATERIALS AND METHODS

Tissue collection and deparaffinization

Tissue samples of the ESCCs and HNSCCs were collected from each patient. Uninvolved lymph nodes were used as normal control tissue for each patient. All formalin-fixed and paraffin-embedded specimens were sectioned into 4- μ m slides and deparaffinized by routine techniques.

Immunohistochemistry for p53, p63, CK5, CK7, and CDX2

The slides were quenched in 0.3% H₂O₂ in methanol for 20 minutes to block endogenous peroxidase. Antigen retrieval was performed in 10 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid buffer (pH 9.0) for 10 minutes at 120°C. After blocking of non-specific binding sites by using serum-free protein-block (X0909, Dako Denmark A/S, Glostrup, Denmark), the slides were incubated with a primary antibody against p53 (DO-7/BP53-12, 1 : 2000 dilution [NeoMarkers, Union City, CA, USA]), p63 (MS-1081-P [4A4], 1 : 400 dilution [NeoMarkers]), CK5 (NCL-L-CK5 [XM26], 1 : 100 dilution, Novocastra, Buffalo Grove, IL, USA), CK7 (MU225-UC [OV-TL12/30], 1 : 400 dilution, Biogenex, Fremont, CA, USA), and CDX2 (MU392A-UC [CDX2-88], 1 : 200 dilution, Biogenex) for 1 hour at room temperature. The slides were then incubated with post-antibody blocking and PowerVision poly-HRP-anti-mouse/ rabbit/rat IgG (Immunologic, Klinipath, Duiven, The Netherlands) for 15 and 30 minutes, respectively. We used 3,3'-diaminobenzidine tetrachloride (DAB) (Sigma D5637, St. Louis, MO, USA) as the chromogen for visualization and hematoxylin for counter-staining. The staining was considered to be positive if more than 50% of the cancerous epithelial cells exhibited dark brown nuclear (p53, p63, cdx2) or cytoplasmic/membranous (CK5, CK7) staining.

DNA isolation

The deparaffinized slides were stained with hematoxylin prior to microdissection. The tumor containing epithelium was outlined and selectively microdissected. Subsequently, the microdissected tissue was subjected to DNA extraction using the QIAamp® DNA Micro Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer's protocol.

TP53 mutation analysis

TP53 mutation analysis was performed for exons 4–9. Eurogentec primers were used (Eurogentec, Maastricht, The Netherlands [sequences are available upon request]). Polymerase chain reaction (PCR) mixes were prepared using Amplitaq DNA Polymerase (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Subsequently, the majority of the PCR reactions were started under the following conditions: (i) 94°C for 3 minutes; (ii) 35 cycles of 94°C for 1 minute, 60°C for 30 seconds, and 72°C for 45 seconds; and (iii) 72°C for 5 minutes. The PCR products were purified with 10 units of exonuclease (Westburg BV, Leusden, The Netherlands) and 1 unit of shrimp alkaline phosphatase (GE Healthcare, Zeist, The Netherlands), and incubated for 30 minutes at 37°C and for 20 minutes at 80°C. The BigDye Terminator cycle sequencing kit (Applied Biosystems) was used for sequencing. The sequencing reaction mix was run on the 3130 genetic analyzer (Applied Biosystems).

LOH analysis

Primers for 10 microsatellite markers were obtained from Eurogentec (Eurogentec). A PCR mix was prepared using Platinum Taq (Applied Biosystems). The PCR was performed under the following conditions (marker-specific conditions are available upon request): (i) 95°C for 5 minutes; (ii) 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute. The analysis was performed using an automated ABI 3130 genetic analyzer (Applied Biosystems), the GeneScan 500 ROX size standard, and the manufacturer's Genescan® 3.1.2 software (Applied Biosystems). By comparing the LOH patterns of the different cancer tissues to normal tissue, the LOH index was calculated. An LOH index of <0.75 or >1.33 was considered as LOH.

RESULTS

Case A

Immunostainings for p63 and CK5 were strongly positive in the EC, whereas CK7 and CDX2 were undetectable in the tumor cells. As expected, CK7 and CDX2 were present in the BE tissue (Fig. 3A–D). These results strongly indicate that the EC is an ESCC and that the tumor did not arise from the BE tissue.

Results of the p53 immunohistochemistry (IHC) are shown in Figure 4A–C. A positive pattern in ESCC and a pattern suspicious for a stop codon (null) mutation in the HNSCC were found. Mutation analysis of the *TP53* gene showed a point mutation in exon 5 (H179N) in the ESCC and a deletion of 14 nucleotides in exon 6 (c.622_635delGACAGAAA CACTTT, p.D208SfsX3) in the HNSCC (Fig. 4D, Table 1), the latter being consistent with abrogated nonfunctional p53 protein. LOH analysis identified loss of two markers in the ESCC only (Table 1). Taken together, these results show that the ESCC was a new primary SCC in BE.

Case B

Expression of p63, CK5, and CK7 was positive in the EC (Fig. 3E–G). CDX2 IHC was negative in the carcinoma and strongly positive in the adjacent BE mucosa (Fig. 3H). CK7 was also present in the BE glands (Fig. 3G), whereas p63 and CK5 were undetectable in BE (Fig. 3E–F). This staining pattern, in combination with the hematoxylin and eosin (H&E), is consistent with a pure ESCC.

IHC for p53 showed a positive staining pattern in all HNSCCs, except for the one at the base of the tongue. A completely negative staining suggestive for a truncating stop codon (null) mutation was seen in the SCC at the base of the tongue and the ESCC (Table 1). The *TP53* mutation analysis showed four different mutations in four of the five SCCs in this patient strongly suggesting that the ESCC was an independent tumor. An insertion of one base pair in codon 255 of exon 7 (c.764_765insT, p.I255IfsX9) in the ESCC resulted in a frame shift and premature termination of the protein, responsible for a nonfunctional p53 protein (Table 1). LOH analysis confirmed that the ESCC in BE was a new primary SCC in BE (Table 1).

DISCUSSION

We report two cases of ESCC arising in association with BE in patients with a previous history of malignancies, i.e. HNSCCs. H&E stainings and IHC for p63, CK5, CK7, and CDX2 together showed that the ECs were indeed ESCCs. There was no evidence for an adenocarcinoma component. In order to investigate whether the ESCCs in BE were primary cancers or metastatic from previously diagnosed HNSCCs, we performed *TP53* mutation analysis. In addition, we studied patterns of allelic loss using 10 microsatellite markers to

validate the strength of LOH analysis as an adjunct to histological examination for distinguishing between metastases of prior cancer and primary cancer. Both analyses showed that the ESCCs in BE in the two cases were indeed *de novo* primary malignancies.

The *TP53* gene is a tumor-suppressor gene located on chromosome 17p, encoding for tumor protein p53. Allelic deletions of the chromosome 17p and point mutations of the *TP53* gene are frequently found in human cancers, and these may disrupt the p53 pathway. Several studies have reported maintenance of identical *TP53* mutations in matched primary and metastatic tumors of the aerodigestive tract.⁶ *TP53* mutations are found in approximately 30–50% of HNSCCs and ESCCs.^{7,8} The most common type of *TP53* mutations in HNSCC is the missense mutation, accounting for 79% of all mutations. Missense mutations are point mutations in which a single nucleotide is replaced, which in most cases result in the substitution of one amino acid for another.⁷ In case B, missense mutations were found in codons 157 and 273.

Immunostaining for p53 is a surrogate marker for mutation of one or both *TP53* alleles. There is usually no or only weak non-diffuse p53 detectable if a carcinoma has two wild-type (non-mutant) alleles. In contrary, tumors that harbor *TP53* missense mutations often show strong and diffuse nuclear accumulation of p53 protein, as the amino acid substitution often results in increased stability of this protein. In case of early abrogation of the p53 protein, either through a nonsense or frame shift (insertion/deletion) mutation, complete absence of p53 expression can be found due to a deletion or inactivation of the non-mutated allele.⁹

The development of cancer types other than EAC in BE is rare. In 1984, Smith *et al.* reported the clinical and pathologic features of 26 patients with EC in BE. Twenty-three patients had EAC (a single lesion in 20 patients and two separate EACs in three patients), one had an ‘adenocarcinoid,’ and one had an adenosquamous carcinoma. Only one patient was diagnosed with an ESCC directly surrounded by BE.¹⁰ The presence of ESCC in BE mucosa has also been reported in two additional patients.^{11,12} Although ESCCs located at or above the squamocolumnar junction has been described several times,^{12–16} to the best of our knowledge, our two patients represent the fourth and fifth reported cases in the English literature with an ESCC detected in BE mucosa. In all likelihood, these SCCs in BE arose from an islet of squamous epithelium that is sometimes seen in BE during upper endoscopy. All five patients were white males, and at least two of these patients had a substantial alcohol and tobacco-smoking history.^{10–12}

Because adenosquamous carcinomas in BE mucosa have been described in the literature,^{10,17–22} we excluded the presence of an adenocarcinoma component in the ECs by reviewing H&E slides and performing IHC for p63, CK5, CK7, and CDX2. There was no evidence for an adenocarcinoma component on the H&E slides. In addition, the squamous epithelium adjacent to the carcinoma revealed high-grade dysplasia/carcinoma *in situ*, which supports the notion that these carcinomas had arisen from squamous mucosa and not from BE mucosa. p63 and CK5 are well-known, highly specific markers for SCCs,^{23,24} and as expected, strong staining was seen in the ECs of both patients. In contrast, CDX2 is often present in BE mucosa and during neoplastic progression of BE toward EAC.^{25,26} In both cases, CDX2 labeling was seen in the BE glands but undetectable in the ECs. This also indicates that both of the ECs had not arisen from BE mucosa. CK7 expression is usually present in the majority of EACs and in a subset (22–29%) of the ESCCs.^{27–29} Therefore, it is not surprising that there was no staining detected in the EC of case A, while the EC of case B exhibited moderate dark staining. Taken together, these data imply that the ECs of our cases are pure ESCCs.

The field cancerization hypothesis was introduced by Slaughter *et al.* in 1953 and suggests that the carcinogenic effects of alcohol and tobacco may simultaneously affect the squamous epithelial mucosa of the oral cavity, pharynx, and upper aerodigestive tract, and stimulate the development of multifocal malignancies.³⁰ Considering the fact that our two patients used substantial amounts of tobacco and alcohol, we believe that the field cancerization hypothesis may have played a role in the development of SCC in these individuals. In addition to their use of tobacco and alcohol, both our patients had received radiation therapy in the head and neck region, a further risk factor for ESCC.³¹

Many studies have shown that patients with HNSCC have an increased risk of developing a second primary ESCC.^{18,19} Patients with a SCC located in the oropharynx and hypopharynx are at a particularly increased risk of ESCC; cumulative risks of 0.98% and 1.90% have been reported at 10 years after diagnosis of oropharyngeal and hypopharyngeal, respectively.³² Therefore, regular surveillance in these patients might be justified. In addition, the presence of columnar metaplasia, as is found in BE, does not preclude the development of ESCC.

In conclusion, it is well established that patients with BE are at an increased risk of developing EAC. We report two cases with primary ESCC arising in the distal esophagus and directly surrounded by BE. Although both BE and ESCC are relatively commonly diagnosed disorders and seem to have similar risk factors, the combination of both diseases in the distal esophagus is surprisingly rarely observed. There may be no association between BE and the development of ESCC, nevertheless, our cases demonstrate that the presence of BE also does not exclude the development of an ESCC in an area of IM. Most probably, the ESCC found in our patients arose from an islet of squamous epithelium in BE, and it is likely that genetic predisposition, field cancerization, and possibly the exposure of the esophagus to radiation therapy played a causative role in the development of ESCC in BE.

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Fig. 1. Upper endoscopy showing a friable, ulcerative esophageal tumor in Barrett's esophagus (BE) in case A. Islets of squamous epithelium were seen within the BE mucosa.

Fig. 2.

Upper endoscopy showing a lesion suspected for a malignancy located in the distal esophagus (case B). The tumor was surrounded by Barrett's esophagus (BE). Areas of normal squamous epithelium were revealed distal to the tumor (in BE).

Fig. 3. p63, CK5, CK7, and CDX2 immunohistochemistry photos (4×) of case A (A–D) and case B (E–H). p63 and CK5 are well-known, highly specific squamous cell carcinoma (SCC) markers. CDX2 is usually present during the neoplastic progression of Barrett's esophagus (BE) toward esophageal adenocarcinoma (EAC) and absent in normal squamous epithelium and SCCs. CK7 is found in the majority of EACs and in a subset (22–29%) of the esophageal SCCs (ESCCs).
Case A: p63 (A) and CK5 (B) were strongly expressed in the ESCC, whereas staining was limited to absent in the adjacent BE mucosa. There was no expression of CK7 (C) and CDX2 (D) detected in the ESCC. In the BE glands, however, expression of both CK7 (C) and CDX2 (D) was revealed.
Case B: p63 (E) and CK5 (F) were overwhelmingly present in the ESCC and undetectable in the surrounding BE glands. Expression of CK7 was found in both the ESCC and the BE mucosa. CDX2 was only revealed in the BE tissue.

Fig. 4.

p53 immunohistochemistry (a–c) and *TP53* mutation analysis (D) results of case A. (A) The black arrows point out the esophageal squamous cell carcinoma (ESCC), and the white arrows show Barrett's esophagus (BE) glands. (B) p53 is strongly expressed in the ESCC arising in association with BE. (C) Absence of expression of p53 in the oral squamous cell carcinoma, indicating a null mutation. In the adjacent normal mucosa, weak staining of the basal cell layer is observed, as usually present, that serves as internal control for proper staining. (D) Deletion of 14 bp in exon 6 of the *TP53* gene, which causes a change in frameshift reading and enables the reading of a stop codon 3 triplets after the first affected amino acid. This eventually results in a shorter, mutant and abrogated nonfunctional protein product.

p53 immunohistochemistry (IHC), *TP53* mutation analysis and loss of heterozygosity (LOH) analysis results of cases A and B

CaseA: IHC for p53 was positive in esophageal squamous cell carcinoma (ESCC) and negative in head and neck squamous cell carcinoma (HNSCC). The *TP53* mutation analysis demonstrated a point mutation in exon 5 in the ESCC and a deletion of 14 base pairs in exon 6 in the HNSCC. LOH analysis only identified loss of two markers in ESCC.

CaseB: IHC for p53 was negative in ESCC and base of tongue HNSCC, and positive in the remaining HNSCCs. The *TP53* mutation analysis showed point mutations in exons 5 and 8 of the oropharynx and palatum durum squamous cell carcinoma (SCC), respectively. An insertion of one base pair in exon 7 and a deletion of 6 base pairs at the end of intron 6 were detected in the ESCC and tongue basis SCC, respectively. No mutation was found in the trigonum retromolare SCC. Although LOH analysis identified loss of at least 6 markers in all SCCs, the LOH patterns observed in these cancers distinguish between the different cancers

Table 1

Case A														
Carcinoma	Year	p53 IHC	TP53 mutation analysis		LOH analysis									
			cDNA	protein	D1S158	D1S228	D3S158	D4S430	D4S1572	D5S644	D9S63	D9S228	D13S158	D18S58
ESCC	2010	Positive	c.535C >A	H179N										
Oral SCC	2007	Negative	c.622_635delGACAGAAACACTTT	p.D208SfsX3										

Case B														
Carcinoma	Year	p53 IHC	TP53 mutation analysis		LOH analysis									
			cDNA	protein	D1S158	D1S228	D3S158	D4S430	D4S1572	D5S644	D9S63	D9S228	D13S158	D18S58
Palatum durum SCC	2009	Positive	c.817C >T	R273C										
ESCC	2008	Negative	c.764_765insT	p.I255IfsX9										
Tongue base SCC	2006	Negative	c.559 + 76_81delTCTTAG	N/A										
Oropharynx SCC	2000	Positive	c.469G > T	V157F										
Trigonum retromolare SCC	2000	Positive	None	None										

( ) LOH; ( ) retention.