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Short Communication

Expression of Bcl-2 and Amplification of *c-myc* Are Frequent in Basaloid Squamous Cell Carcinomas of the Esophagus

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Basaloid squamous cell carcinoma (BSCC) of the esophagus is a rare, poorly differentiated variant of typical esophageal squamous cell carcinoma (SCC) characterized by high proliferative activity and frequent spontaneous apoptoses. In the present study, we investigated the expression of the apoptosis-suppressing protein Bcl-2 in 23 BSCC of the esophagus and 23 stage-matched typical esophageal SCC by means of immunohistochemistry. In addition, amplification of the apoptosis- and proliferation-inducing gene *c-myc* was determined by means of differential polymerase chain reaction. Bcl-2 expression was found significantly more often in BSCC than in SCC (86.9% vs. 17.4%, $P < 0.0001$). Amplification of *c-myc* was nearly twice as common in BSCC as in SCC (47.8% vs. 26.1%, not significant). Bcl-2 protein expression together with c-myc amplification was detected in 43.5% of the BSCC but in none of the typical SCC ($P < 0.0001$). Taken together, our findings indicate that the molecular pathogenesis of esophageal BSCC differs from that of typical SCC and frequently involves co-activation of *c-myc* and Bcl-2. *(Am J Pathol 1999, 155:1027–1032)*

Basaloid squamous cell carcinoma (BSCC) is an uncommon variant of squamous cell carcinoma (SCC) that arises in a variety of anatomic sites, including the upper aerodigestive tract,[1–3] the anus,[4] the thymus,[5] and the uterine cervix.[6] Histologically, BSCC is defined as an invasive carcinoma composed of closely packed cells with hyperchromatic nuclei and scant cytoplasm. The tumors display a predominantly solid growth pattern, small cystic spaces, and foci of comedo-type necrosis. Additionally, BSCC is intimately associated with dysplastic squamous epithelium, *in situ* SCC, invasive SCC, or foci of SCC among basaloid cells.[1] Recently, we described the histological and clinical features of a series of BSCC of the esophagus.[7] In this investigation, we were able to show that esophageal BSCC are characterized by significantly higher proliferative activity and a significantly higher rate of spontaneous apoptosis than typical SCC. In another study on the expression of the apoptosis-regulating protein Bcl-2 in esophageal SCC, we had observed that expression of Bcl-2 is more common in poorly differentiated SCC of the esophagus than in well differentiated SCC.[8] We also noted that expression of Bcl-2 was especially strong in BSCC (unpublished results). A similar observation has recently been published by Koide et al,[9] who found strong expression of Bcl-2 protein in a small series of 4 BSCC of the esophagus.

The simultaneous finding of high apoptotic rate and strong expression of Bcl-2 in BSCC is difficult to understand, given that Bcl-2 is known to function as a suppressor of apoptosis.[10] We therefore speculated that the frequent overexpression of Bcl-2 in BSCC must be counteracted by activation of one or more pro-apoptotic and/or proliferation-promoting proto-oncogenes. The proto-oncogene *c-myc* seemed to be a very promising candidate in this context. Thus, much *in vitro* and *in vivo* data indicate that increased expression of *c-myc* blocks differentiation[11–14] and enhances proliferative and apoptotic activity.[15–17] Moreover, it has been shown in experimental tumor systems that increased expression of Bcl-2 and *c-myc* can cooperate in tumorigenesis.[18–21]

In the present study, we therefore determined the expression of Bcl-2 protein and screened for amplification of the c-myc gene in a series of 23 BSCC of the esophagus and compared the results with those obtained for 23 stage-matched typical SCC of the esophagus. Our results indicate that Bcl-2 overexpression and *c-myc* am-

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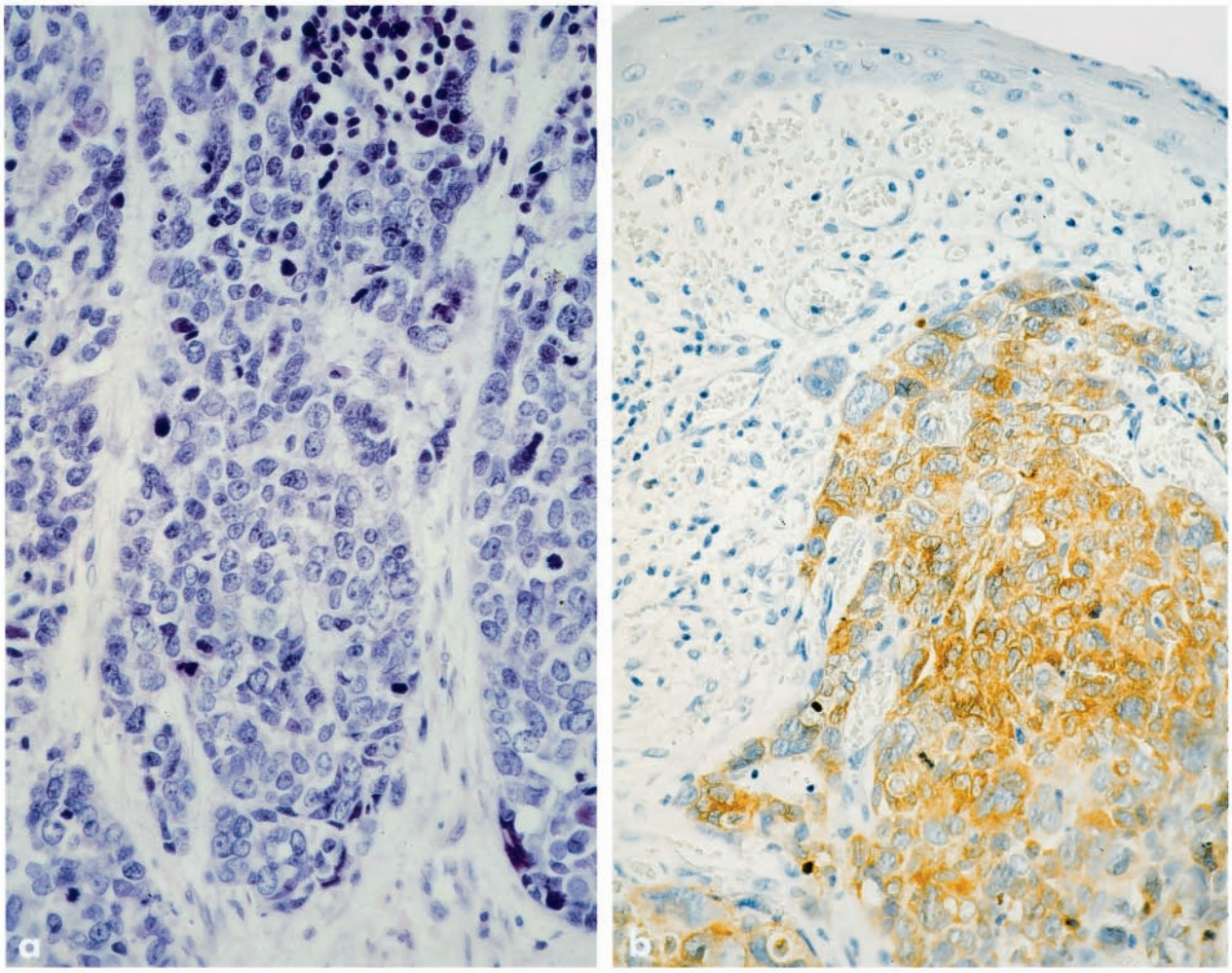
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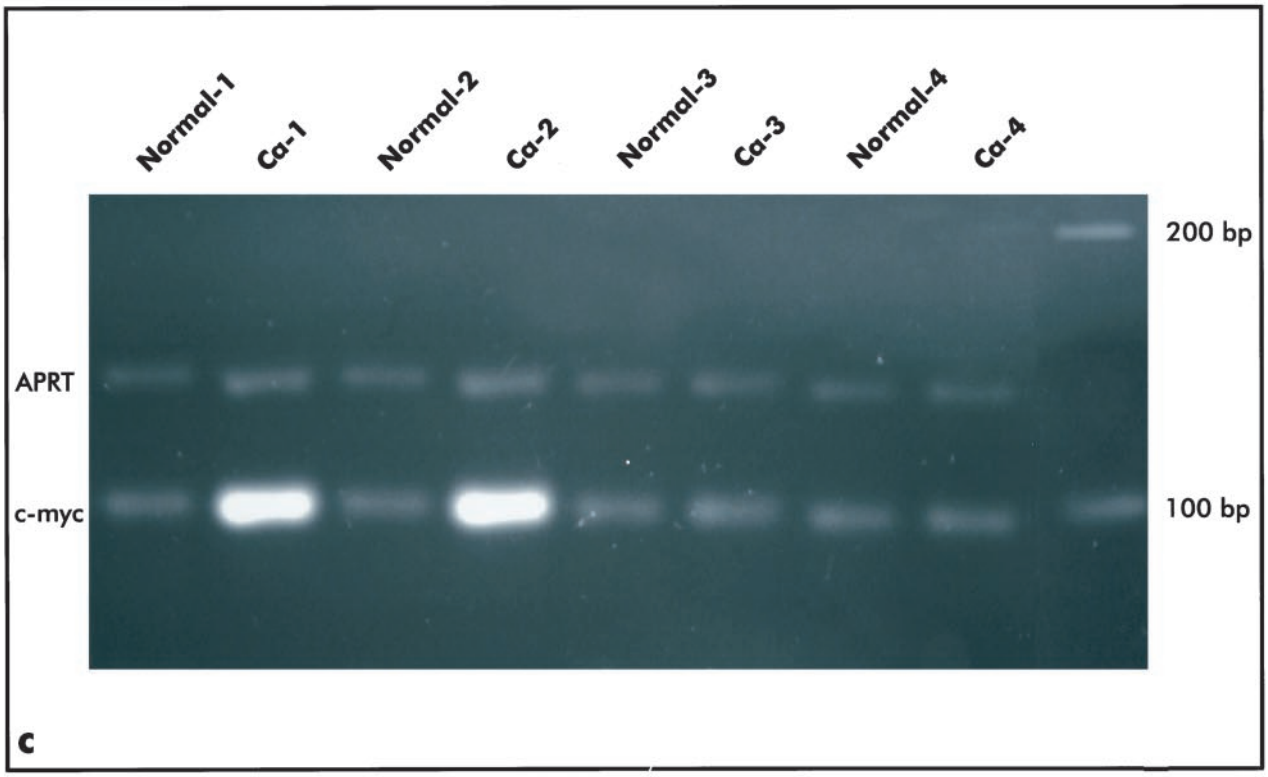
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APRT
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plification frequently occur in BSCC but not in SCC, a finding suggesting a cooperative function of Bcl-2 and *c-myc* in the molecular pathogenesis of esophageal BSCC.

Materials and Methods

Specimen Selection

Twenty-three basaloid squamous cell carcinomas of the esophagus, classified according to the criteria of Wain et al,[1] were retrieved from the files of the Institutes of Pathology of the Universities of Düsseldorf and Mainz, Germany. As a control, 23 stage-matched typical squamous cell carcinomas of the esophagus were selected. All tumors had been resected between 1978 and 1998 without prior radio- or chemotherapy.

The 23 BSCC were from 19 male and 4 female patients. The median age at operation was 61 years (range, 45–72 years). The typical SCC were from 14 male and 9 female patients (median age 59 years; range, 42–67 years).

Pathological Review

The surgical specimens were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The tumor stage was determined according to the criteria proposed by the UICC.[22] The grade of tumor differentiation of the 23 typical SCC was determined according to the criteria proposed by the World Health Organization.[23] Accordingly, 5 BSCC were in stage I, 4 were in stage IIA, 2 were in stage IIB, and 12 were in stage III. The distribution of the 23 typical SCC according to tumor stage was identical to that of the BSCC. Of the typical SCC, 2 were graded as G1, 11 as G2, and 10 as G3. No grading was performed for the BSCC because there is currently no generally accepted grading system for this tumor type.

Bcl-2 Immunohistochemistry

For each carcinoma, one representative block including central and peripheral portions of the tumor was selected. After microwave pretreatment, immunostaining was performed using the monoclonal antibody 124 (1:40; Dako, Glostrup, Denmark) as described previously.[8] Negative controls were performed by replacing the primary antibody by an irrelevant isotype-matched monoclonal mouse antibody at the same dilution as the Bcl-2 antibody. Tonsillar tissue was used as positive control. In addition, positive staining of tumor-infiltrating lymphocytes provided an internal control for Bcl-2 staining.

The percentage of Bcl-2-positive tumor cells was determined semiquantitatively by assessing the entire tumor section. Each sample was assigned to one of the following categories: 0 (0–4%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 (75–100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), or 2+ (strong). Staining intensity was judged relative to lymphocytes within the sample, which were designated arbitrarily as 2+.[24] Finally, an immunoreactive score was calculated by multiplying the percentage of positive cells by the staining intensity score, as proposed by Krajewska et al.[25] In the case of heterogeneous staining intensities within one sample, each component was scored independently and the results were summed. For example, a specimen containing 25% tumor cells with strong intensity ($1 \times 2+ = 2$), 25% tumor cells with weak intensity ($1 \times 1+ = 1$) and 50% tumor cells without immunoreactivity received a score of $2 + 1 + 0 = 3$.

Differential PCR Analysis for c-myc Amplification

For DNA preparation two 10-μm slices from paraffin blocks containing tumor tissue and adjacent normal tissue (eg, lymph-node tissue or gastric tissue from the distal resection margin) were dewaxed and lightly stained with hematoxylin. Subsequently, tumor tissue and normal tissue were dissected from the slides under light microscopic control and placed into reaction cups containing TE buffer (10 mmol/L Tris-Cl; pH 7,5; 0,1 mmol/L EDTA). After proteinase K digestion overnight (1 mg/ml; 55°C), and inactivation of proteinase K (94°C, 8 minutes), the preparations were used for PCR without further purification.

For differential PCR analysis,[26] a 96-bp fragment of the *c-myc* gene (target gene) located on chromosome 8 was coamplified with a 134-bp fragment of the *APRT* (adeninphosphoribosyl-transferase) gene (control gene) located on chromosome 16. The primer sequences were 5′ - CCT CAA CGT TAG CTT CAC CAA C-3′ and 5′-CTG CTG GTA GAA GTT CTC CTC - 3′ for *c-myc*, and 5′ - TGG GAA AGC TGT TTA CTG GC - 3′ and 5′ - CAG GGA ACA CAT TCC TTT GC - 3′ for *APRT*. Differential PCR was performed in a final volume of 50 μl with 2 μl of DNA template, PCR buffer containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 30 pmol primer for the *c-myc* gene, 60 pmol primer for the *APRT* gene, and 2 U Taq DNA-polymerase (PCR Core Kit, Qiagen, Hilden, Germany). Initial denaturation at 94°C for 5 minutes was followed by 30 cycles on a thermocycler (UNO; Biometra, Göttingen, Germany). These included denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1 minute. A final extension step at 72°C was performed for 4 minutes.

Figure 1. Typical light microscopic aspect of a basaloid squamous cell carcinoma (BSCC) composed of cells with hyperchromatic nuclei and scant cytoplasm (**a**); H&E; original magnification, ×400. Strong expression of Bcl-2 protein (brown cytoplasmatic reaction product) in an esophageal BSCC. Note the absence of Bcl-2 expression in the neighboring nonmalignant esophageal squamous epithelium (**b**); original magnification, ×400. Ethidium bromide-stained agarose gel of a differential PCR for the *c-myc* gene and the *APRT* gene in carcinoma tissues (Ca) and corresponding normal tissues (Normal) from 4 cases of esophageal BSCC. Note amplification of the *c-myc* gene in tumors 1 and 2, no *c-myc* amplification in tumors 3 and 4 (**c**).

As a positive control, we used DNA from the colon carcinoma cell line COLO320DM, previously shown to have amplified *c-myc*[27] and DNA from a formalin-fixed, paraffin-embedded esophageal SCC with known *c-myc* amplification. Peripheral leukocyte DNA and normal placental DNA were used as reference templates with normal gene copy number.

PCR products were separated on 3% agarose gels, and the ethidium-bromide-stained bands were recorded by the Gel-Doc 1000 system (Bio-Rad, München, Germany). Quantitative densitometric evaluation of the target gene signal intensity relative to the control gene signal intensity was performed using Molecular Analyst software, version 2.1 (Bio-Rad, München, Germany). Only increases in the target gene/control gene quotients more than 3 times that of corresponding normal tissue were considered as evidence of gene amplification.[28,29]

Tumors with evidence of *c-myc* amplification in the initial analysis were retested by performing a second independent PCR. In addition, differential PCR analysis using a second control gene locus was performed in all these cases. Therefore, a 82-bp fragment of the *IFNG* (γ-interferon) gene, located on chromosome 12, was co-amplified with the 96-bp fragment of the *c-myc* gene. The primer sequences were 5′ - GCA GAG CCA AAT TGT CTC CT - 3′ and 5′ - GGT CTC CAC ACT CTT TTG GA - 3′ for *IFNG*. The PCR conditions were identical to those for the PCR with *APRT* as control gene, except that the quantities of primer were 60 pmol for *c-myc* and 30 pmol for *IFNG*.

Statistical Analysis

Statistical analysis was performed using the SAS software package (SAS Institute Inc., Cary, NC). Correlations between the expression of Bcl-2, amplification of *c-myc*, and tumor type were analyzed by means of the two-sided Fisher's exact test; P values <0.05 were considered significant.

Results

Expression of Bcl-2 in Normal Esophageal Tissue

In normal esophageal squamous epithelium adjacent to the carcinomas, cytoplasmatic Bcl-2 immunoreactivity was found in the basal cell layer, whereas suprabasal cells showed no Bcl-2 immunoreactivity. The intensity of Bcl-2 staining was weak (1+) compared to lymphocytes within the same section. In addition, Bcl-2 expression was found in neurons of the myenteric plexus as well as in smooth muscle cells of the arterial walls, the muscularis mucosae and the muscularis propria.

Expression of Bcl-2 in BSCC and in SCC

Twenty out of 23 BSCC (Fig. 1a) showed cytoplasmatic expression of Bcl-2 (Fig. 1b), 2 with weak (1+), and 18 with strong (2+) staining intensity, whereas 3 BSCC were completely negative. Regarding the percentage of positive tumor cells, 2 were in category 1 (5–24%), 4 in category 2 (25–49%), 7 in category 3 (50–74%), and 7 in category 4 (75–100%). The immunoreactive scores of the 20 Bcl-2-positive BSCC ranged between 2 and 8 (median, 5).

In contrast, only 4 SCC showed cytoplasmatic immunoreactivity for Bcl-2; 19 cases were negative. The 4 Bcl-2-positive SCC showed only weak (1+) staining intensity. With regard to the percentage of positive cells, all positive cases were in category 1 (5–24%); the immunoreactive score of each of the 4 Bcl-2-positive SCC was 1.

Upon comparison of Bcl-2 expression in BSCC and SCC (negative vs. positive), the difference between the two groups was highly significant ($P < 0.0001$; Fisher's exact test).

Amplification of c-myc *in BSCC and SCC*

Amplification of *c-myc* was found in 11 out of 23 BSCC and in 6 out of 23 SCC (Fig. 1c). Differential PCR analysis using *APRT* as control gene showed results identical to those for differential PCR using *IFNG* as control gene. None of the corresponding normal tissues under investigation showed evidence of *c-myc* amplification. Comparison of the frequency of *c-myc* amplification in BSCC and SCC revealed no significant difference (Fisher's exact test).

Amplification of c-myc *and Expression of Bcl-2 in BSCC and in SCC*

Both aberrations, amplification of *c-myc* and expression of Bcl-2, were simultaneously detected in 10 BSCC but in none of the SCC under investigation. This difference was significant according to Fisher's exact test ($P < 0.0001$).

Discussion

The present study shows that expression of Bcl-2 protein is more frequent and stronger in BSCC than in typical SCC of the esophagus. In addition, we have demonstrated that amplification of *c-myc* is nearly twice as common in BSCC as in typical SCC. Both aberrations, ie, expression of Bcl-2 and amplification of c-myc, were present in nearly half of the BSCC but in none of the SCC in this investigation. These data suggest that coactivation of Bcl-2 and c-myc may be important for the pathogenesis of esophageal BSCC. Prior evidence of cooperation between c-myc and Bcl-2 derives mainly from experimental data. For example, induction and progression of malignant lymphomas through cooperation of Bcl-2 and c-myc has repeatedly been shown in *c-myc/Bcl-2* double-transgenic mice.[19–21] A similar effect was found for the transformation of cultured fibroblasts[30] and cultured bone marrow cells.[18] Mechanistically, this cooperation is explained by a Bcl-2-induced suppression of the c-myc-induced apoptosis without blockade of the proliferation-

inducing effect of c-myc.[21,30–32] In contrast, evidence of cooperation between c-myc and Bcl-2 in clinical tumor samples is still limited. Thus, Wang et al[33] found coexpression of Bcl-2 and c-myc in the majority of pheochromocytomas. In neuroblastomas, a cooperative effect between Bcl-2 and the c-myc homologue N-myc has been suggested.[34] Except for one study on medullary thyroid cancer,[35] the possible coordinate aberration of Bcl-2 and c-myc in human carcinomas has not yet been investigated. This may be partly attributable to technical problems. Due to DNA degradation in formalin-fixed tumor samples, determination of *c-myc* amplification is not possible by Southern blot analysis. On the other hand, immunohistochemical analysis of c-myc protein expression has produced inconclusive results due to the short halftime of the c-myc protein[36] and the negative effects of formalin fixation on antigenicity.[37] Against this background, differential PCR analysis provides a reliable alternative method for the detection of *c-myc* amplification in paraffin-embedded tumor samples.[26,28,29] This can be appreciated from the fact that the frequency of *c-myc* amplification found in our series of typical SCC corresponds well with previously published Southern blot-based figures on the frequency of *c-myc* amplification in this tumor type.[38]

Genetic alterations involved in the development of BSCC, especially BSCC of the esophagus, are largely unknown. Recently, Abe et al[39] have demonstrated DNA aneuploidy by means of DNA image cytometry in 100% of a small series of 7 esophageal BSCC. The main reason for the current lack of information on the molecular pathogenesis of BSCC may be that it is a relatively rare and only recently recognized variant of typical SCC. Thus, only 43 cases of esophageal BSCC, including our 23 cases, have been published to date.[7,9,39–43] Our study addresses for the first time the analysis of molecular genetic alterations in esophageal BSCC. We demonstrate that these tumors frequently show Bcl-2 overexpression and *c-myc* amplification. With regard to the temporal sequence of these aberrations, however, we cannot conclude from our data whether amplification of *c-myc* follows overexpression of Bcl-2 in the development of BSCC or vice versa. However, the first possibility seems to be more likely, because overexpression of Bcl-2 was more frequent than *c-myc* amplification. This hypothesis is also supported by our previously published observation that Bcl-2 overexpression may already occur in precursor lesions of esophageal cancer (ie, severe squamous dysplasias, carcinomas *in situ*).[8] The potential role of Bcl-2 in the development of tumors has been explained by its apoptosis-suppressing effect, which provides a growth advantage to Bcl-2-overexpressing cells and allows the accumulation of oncogenic mutations during a prolonged life span.[44] However, our data indicate that Bcl-2 overexpression may only incompletely protect malignant cells in BSCC from apoptosis because these tumors, in spite of their frequent Bcl-2 overexpression, are characterized by higher rates of spontaneous apoptoses than typical SCC.[7] Although the increased expression of c-myc due to gene amplification may at least partly account for this phenomenon, its precise molecular basis remains to be elucidated. Nevertheless, our data indicate that alterations other than concomitant Bcl-2 overexpression and *c-myc* amplification must be involved in the development of BSCC, insofar as only 10 of the 23 cases under investigation showed both aberrations. In this context it is possible that other mechanisms for *c-myc* activation than gene amplification may play a role in BSCC, eg, chromosomal translocation[21] or transcriptional upregulation by increased expression of activating transcription factors.[17,45] Additionally, other genes may play an important role. Future investigations on mutations in the *p53* gene would be especially interesting, because it well known that *p53* not only plays an important role in the regulation of proliferation and apoptosis but also is frequently mutated in typical esophageal SCC.[38]

In conclusion, our data provide evidence for the first time that the molecular pathogenesis of esophageal BSCC differs from that of typical SCC and frequently involves coactivation of *c-myc* and Bcl-2.

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