Neoplasia • Vol. 8, No. 9, September 2006, pp. 702–707

www.neoplasia.com

c-myc Amplification Is Frequent in Esophageal Adenocarcinoma and Correlated with the Upregulation of *VEGF-A* Expression[1]

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Abstract

BACKGROUND: **Deregulation of *c-myc* plays a major role in the carcinogenesis of human malignancies. We investigated the amplification of the *c-myc* gene in a surgical series of Barrett cancers. *METHODS:* Primary resected esophageal (Barrett) adenocarcinomas (*n* = 84) were investigated for *c-myc* amplification using chromogene *in situ* hybridization. Tumor samples were assembled in a tissue microarray. *c-myc* gene dosage was correlated with clinicopathologic parameters, including the survival and gene expression of cyclooxygenases (*COX-1* and *COX-2*) and proangiogenic growth factors (*VEGF-A* and *VEGF-C*). *RESULTS:* The majority (70 of 84; 83.3%) exhibited amplification of the *c-myc* gene. There were low-level amplifications in 63 (75.0%) cases and high-level amplifications in 7 (8.3%) cases. No amplification was found in 14 (16.7%) cases. Tumors without *c-myc* amplification had lower *VEGF-A*, *VEGF-C*, and *COX-2* expression levels than tumors with low-level and high-level *c-myc* amplification (statistically significant for *VEGF-A*; *P* = .0348). *c-myc* amplification was not correlated with clinicopathological parameters or survival. Only diffuse and mixed-type tumors, according to Lauren classification, exhibited *c-myc* amplifications more frequently (*P* = .0466). *CONCLUSIONS:* Amplifications of the *c-myc* gene are frequent in Barrett cancer. *c-myc* may be involved in the regulation of angiogenesis.**

Neoplasia (2006) **8,** 702–707

Keywords: c-myc, esophageal adenocarcinoma, Barrett cancer, angiogenesis, VEGF.

Introduction

Barrett cancers comprise an entity of increasing clinical importance due to a vastly unexplained rapid rise in incidence in recent decades [1,2]. Chances for cure are still limited to surgical resection at an early stage of the disease [3,4]. Histologically, these esophageal tumors are adenocarcinomas arising in the distal esophagus within the precancerous Barrett esophagus, which is defined as a metaplastic change of the normal esophageal squamous mucosa [2]. Malignant progression is driven by the chronically damaging effect of gastroesophageal reflux, which promotes a characteristic histopathologic sequence, from specialized intestinal metaplasia to low-grade and high-grade intraepithelial neoplasia to invasive adenocarcinoma [2]. Despite intensive research, knowledge about molecular mechanisms underlying Barrett cancer is still rather limited [5].

The proto-oncogene *c-myc* ("cellular myomatosis oncogene") encodes a transcription factor that is regarded essential for progression in human malignancies. It is located at the chromosomal region 8q23–24 [6]. The *c-myc* gene has also been implicated in Barrett carcinogenesis. Amplification of the gene *c-myc* [7,8], as well as overexpression of the c-myc oncoprotein [9], has been demonstrated in invasive Barrett adenocarcinomas, but with great variability (Table 1). Investigations of precursor lesions (high-grade intraepithelial neoplasia, which has been previously addressed as "dysplasia") [10] have suggested that *c-myc* dysregulation by amplification is an early event during Barrett carcinogenesis [7–9] because it is frequently found also in precursor lesions of esophageal adenocarcinoma, especially of high-grade intraepithelial neoplasia.

c-myc has been implicated in various cellular processes, including cell growth, proliferation, loss of differentiation, apoptosis [11,12], and regulation of angiogenesis [13]. Angiogenesis is regarded as an essential feature of malignant tumors. Only recently, we have demonstrated the importance of proangiogenic factors (*VEGF-A* and *VEGF-C*) and their regulation by the prostaglandin biosynthetic pathway (*COX-1* and *COX-2*) in Barrett cancer [14].

It has been estimated that *c-myc* is associated with poor prognosis and has been, therefore, suggested as a clinically relevant marker [12]. This has been shown for c-myc expression in colorectal carcinomas [15]. Moreover, inactivation of

Abbreviations: SSC, standard saline citrate

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[1]This work was supported by Deutsche Krebshilfe (grant no. 70-2789-Si3).

Received 5 April 2006; Revised 21 June 2006; Accepted 24 June 2006.

DOI 10.1593/neo.06277

Table 1. Frequency of *c-myc* Amplification/Overexpression in Barrett Adenocarcinomas Based on Available Literature.

Publication	*n*	Method	Frequency of c-myc Amplification/Overexpression
Investigation of the amplification of the c-myc gene in the 8q23 chromosomal region			
Miller et al. [8]	87	Southern blot analysis	4 of 87 (4.6%)
Sarbia et al. [7]	43	Differential PCR	17 of 39 (43.6%)
Arnould et al. [18]	15*	Comparative genomic hybridization (CGH)	Gain of 8q24 in 8 of 15 cases (53.3%)
Bhargava et al. [19]	28†	CGH	Gain of 8q in 79% recurrent high-level amplifications of 8q23–24.1 in > 10%
Walch et al. [6]	30	CGH	High-level amplification of 8q23–24 in 24 of 30 cases (80%)
Current investigation	84	CISH	70 of 84 (83.3%)
Investigation of c-myc protein overexpression			
Tselepis et al. [9]	20	Western blot analysis	18 of 20 (90%)

*Adenocarcinomas of the esophagus or gastroesophageal junction. No further information is given in the paper. Thus, this paper does not deal with pure Barrett cancer collective data.
†Only 11 adenocarcinomas of the distal esophagus. The remaining 17 tumors under investigation were adenocarcinomas located at a lower level of the esophagogastric junction (not Barrett cancers).

c-myc, or downstream targets of c-myc, might provide important therapeutic targets [16].

Study Aims

We have investigated the frequency of *c-myc* amplification in a large series of primary resected Barrett cancers using chromogene *in situ* hybridization (CISH). We aimed at the following:

- To clarify controversial results on the frequency of *c-myc* amplifications, with previous studies reporting different results in this respect [7,8], using a highly sensitive CISH method
- To elucidate whether *c-myc* amplification is correlated with the expression of genes involved in angiogenesis, which we have investigated previously in a subset of tumors included in the current study [14]
- To analyze the correlation of *c-myc* amplifications with clinicopathological parameters, including survival.

Materials and Methods

Patients

One hundred thirty-seven primary resected esophageal adenocarcinomas arising in association with Barrett epithelium were investigated. Due to technical reasons associated with CISH (described below), only 84 cases were eligible for the final analysis. Patient and tumor characteristics, such as age, gender, tumor size, pT/pN/pL category, Union Internationale Contre le Cancer (UICC) stage [17], and Lauren classification and grading [10], are included in Table 2.

All patients had undergone primary surgical resection (radical transthoracic or transhiatal esophagectomy with lymphadenectomy) at the Technical University of Munich between 1991 and 2003. None of these patients had received prior antineoplastic therapy (neither chemotherapy nor radiochemotherapy). Patients' approval was secured according to local arrangements by the ethics committee. This study was performed with patients' consent allowing molecular research to be performed on specimen obtained during surgical resection.

Tissue Array

The tumor tissues of the 137 esophageal adenocarcinomas under analysis were assembled in tissue microarrays. Core needle biopsies were retrieved from original tumor blocks using a manual arrayer (Beecher Instruments, Sun

Table 2. Level of *c-myc* Amplification in the Whole Population (*n* = 84 Eligible Primary Resected Barrett Cancer Cases Investigated with CISH) and in Subgroups.

	n	No Amplification [*n* (%)]	Low-Level Amplification [*n* (%)]	High-Level Amplification [*n* (%)]	*P* (Chi-Square Analysis)
Whole population	84	14 (16.7)	63 (75.0)	7 (8.3)	
Subgroups					
Age (years)					
< 64	45	6 (13.3)	34 (75.6)	5 (11.1)	ns
> 64	39	8 (20.5)	29 (74.4)	2 (5.1)	
Sex					
Male	78	14 (18.0)	57 (73.1)	7 (9.0)	ns
Female	6	0	6 (100.0)	0	
Tumor size* (mm)					
< 50	52	7 (13.5)	38 (75.0)	6 (11.5)	ns
> 50	32	6 (18.8)	25 (78.1)	1 (3.1)	
Depth of invasion (pT category)					
pT1/2	47	8 (9.5)	34 (40.5)	5 (6.0)	ns
pT3/4	37	6 (7.1)	29 (34.5)	2 (2.4)	
Lymph node involvement (pN category)					
pN0	40	8 (20.0)	28 (70.0)	4 (10.0)	ns
pN1	44	6 (13.6)	35 (79.6)	3 (6.8)	
Lymphatic vessel invasion (L category)					
L0	59	11 (18.6)	45 (76.3)	3 (5.1)	ns
L1	25	3 (12.0)	18 (72.0)	4 (16.0)	
UICC stage					
I/II	55	9 (16.4)	40 (72.7)	6 (10.9)	ns
III/IV	29	5 (17.2)	23 (79.3)	1 (3.5)	
Differentiation (grade)					
G1/2	41	6 (14.6)	33 (80.5)	2 (4.9)	ns
G3/4	43	8 (18.6)	30 (69.8)	5 (11.6)	
Lauren classification					
Diffuse	2	0	1 (50.0)	1 (50.0)	.0466
Intestinal	74	14 (18.9)	56 (75.7)	4 (5.4)	
Mixed	8	0	6 (75.0)	2 (25.0)	

*Size range: 5 to 130 mm.

Prairie, WI) and positioned in a recipient paraffin array block. Viable representative areas of tumor specimens were marked by an experienced pathologist (M.S.). At least three tissue cylinders with a diameter of 0.6 mm were obtained from each tumor block.

CISH

Four- to 5-μm-thick sections were cut from microarray paraffin blocks and dewaxed with xylene, and then rehydrated with 100% ethanol and water. Target retrieval and enzyme digestion were achieved using a commercially available tissue pretreatment kit (no. 00-8401; from Zymed Laboratories, distributed through Zytomed, Berlin, Germany). The sections were dehydrated in upgrading ethanol series and air-dried. Fifteen microliters of digoxigenin-labeled *c-myc* probe (84-1700; from Zymed Laboratories, distributed through Zytomed) was applied on the microarray section, enclosed by a coverslip, sealed, and codenatured on a hot plate (Hybrite; from Vysis, distributed through Abbott, Wiesbaden, Germany) for 5 minutes at 94°C. Hybridization was performed overnight at 37°C. Then, the coverslip was removed by soaking in standard saline citrate (SSC) solution at room temperature and washed in SSC for 5 minutes at 75°C. The remaining hybridized probe linked to digoxigenin was detected by mouse antidigoxigenin antibody followed by polymerized horseradish peroxidase–goat antimouse immunoglobulin. Peroxidase was developed with diaminobenzidine, and nuclei were counterstained with hematoxylin. All detection reagents were provided in commercially available kits (Spotlight CISH Polymer Detection Kit, 84-9246; from Zymed Laboratories, distributed through Zytomed).

The interpretation of CISH results was performed by a senior pathologist (M.S.) with a light microscope using a ×40 objective (original magnification, ×400; Figure 1, *A* and *B*). Signals were seen as nuclear dark brown dots. Fifty to 100 nonoverlapping tumor cell nuclei were evaluated per sample. A gene copy number of one to five copies per nucleus was scored as "no amplification" (Figure 1*A*). A gene copy number of 6 to 10 copies per nucleus in at least 50% of cancer cells was considered "low-level amplification." A gene copy number of more than 10 copies per nucleus or the presence of clusters in at least 50% of cancer cells was considered as "high-level amplification" (Figure 1*B*) [18,19]. Non-neoplastic cells in tissues were always evaluated as intest quality controls.

Due to various reasons (i.e., loss of tissue cores during hybridization procedures, lack of hybridization, or lack of viable tumor tissues in tissue cores), only 84 of the initial 137 esophageal adenocarcinoma cases could finally be assessed for *c-myc* gene dosage.

Results

c-myc gene amplification was assessed using tissue arrays that included 137 esophageal adenocarcinomas. Due to various technical reasons (i.e., loss of tissue cores during hybridization procedures, lack of hybridization, or lack of viable tumor tissue in tissue cores), only 84 of the initial 137 esophageal adenocarcinoma cases could be finally assessed for *c-myc* gene dosage. The majority of cases (70 of 84; 83.3%) exhibited amplification of the *c-myc* gene. These were low-level amplifications in 63 (75.0%) cases and high-level amplifications in 7 (8.3%) cases. No amplification was found in 14 (16.7%) cases (Table 2).

Subgroup analysis according to clinicopathological parameters (Table 2) did not show any statistically significant differences with respect to *c-myc* amplification (patient age, sex, tumor size, pT/pN category, lymphatic vessel invasion, UICC stage, and tumor grade), except for Lauren classification ($P = .0466$). Thus, none of the diffuse or mixed-type tumors according to Lauren classification lacked *c-myc* amplifications. Six (75.0%) of eight mixed-type tumors under investigation and one (50.0%) of two diffuse-type tumors exhibited low-level amplifications of the *c-myc* gene. Furthermore, two (25.0%) of eight mixed-type tumors and one (50.0%) of two diffuse-type tumors showed high-level amplifications of c-myc.

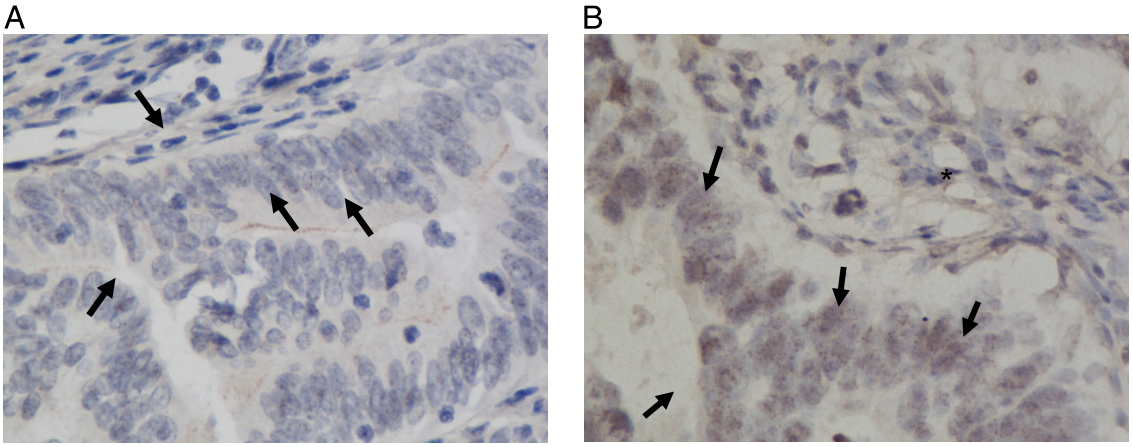

Figure 1. *(A) Cells of a moderately differentiated adenocarcinoma (center and bottom) with a maximum of five nuclear brown dots (arrows indicate nuclei) indicating no evidence of c-myc gene amplification (original magnification, ×600). (B) In contrast, another moderately differentiated adenocarcinoma (bottom left) with more than 10 dots per nucleus (arrows indicate nuclei) indicating high-level amplification of the c-myc gene (original magnification, ×600). Adjacent stromal cells show no evidence of c-myc gene amplification (asterisk).*

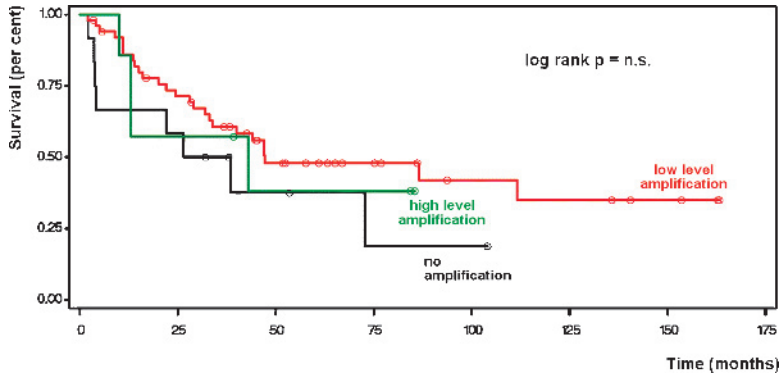

Figure 2. *c-myc amplifications did not significantly affect outcome after the resection of Barrett cancer (univariate survival analysis according to the Kaplan-Meier method).*

Survival analysis according to the univariate Kaplan-Meier method did not show any significant impact of *c-myc* amplification on patients' survival (Figure 2).

In a subset of tumors under analysis ($n = 51$), data on the mRNA expression of four genes [i.e. cyclooxygenases (*COX-1* and *COX-2*) and proangiogenetic growth factors (*VEGF-A* and *VEGF-C*), which were determined in a previous investigation] [14] were available. Correlation analysis showed that tumors without *c-myc* amplification tended to have lower *COX-2*, *VEGF-A*, and *VEGF-C* mRNA expression levels than tumors with low-level amplification, which had lower expression levels compared to cases with high-level amplification (Table 3). This trend toward increased gene expression with increasing level of *c-myc* amplification was statistically significant for *VEGF-A* ($P = .0466$; chi-square analysis).

Table 3. Correlation of *c-myc* Amplification Data with Relative mRNA Expression Levels of *COX-1*, *COX-2*, *VEGF-A*, and *VEGF-C*, as Determined by Quantitative Reverse Transcription PCR (*TaqMan*).

	*n**	No Amplification	Low-Level Amplification	High-Level Amplification	*P* (Two-Sided Jonckheere-Terpstra Test)
COX-1					
Mean	51	12.18	13.65	9.36	ns
SD		16.03	23.48	4.73	
Median		8.13	3.85	9.51	
Min		0.05	0.01	3.57	
Max		49.47	108.11	15.57	
COX-2					
Mean	51	12.98	24.36	27.03	ns
SD		13.96	42.87	19.77	
Median		6.22	10.14	16.15	
Min		0.82	0.05	7.37	
Max		39.25	259.63	59.32	
VEGF-A					
Mean	51	3.22	7.93	9.841	.0348
SD		3.29	15.92	7.92	
Median		2.88	3.71	7.78	
Min		0.08	0.08	1.88	
Max		11.99	104.57	22.80	
VEGF-C					
Mean	51	0.06	0.08	0.15	ns
SD		0.08	0.17	0.11	
Median		0.01	0.00	0.14	
Min		0.00	0.00	0.01	
Max		0.22	1.14	0.36	

*The number of cases varies according to the eligibility of available information (CISH and gene expression analysis with *TaqMan*).

Discussion

According to our current investigation, amplification of the *c-myc* gene is frequent in esophageal adenocarcinomas. However, the majority of cases showed low-level amplifications (75% of the cases), whereas only a minority of the tumors (7.3%) showed high-level amplifications. Only at first glance do these findings contradict existing data in the literature (Table 1). Thus, Miller et al. [8], investigating a series of similar size (87 Barrett cancers) for c-myc amplification, found amplification of *c-myc* in only 4 (4.6%) cases. However, this discrepancy can be well explained by the fact that Miller et al. used Southern blot analysis, which has a considerably lower sensitivity than CISH. In contrast, comparative gene expression data have also shown that genetic gains are frequent at 8q.23–24, the chromosomal region where c-myc is localized (Table 1). Walch et al. [6] demonstrated high-level amplification of the region in 80% (24 of 30) Barrett cancer cases. Moskaluk et al. [20] found gains of 8q24 in 53.3% (8 of 15) cases, whereas van Dekken et al. [21] found gains of 8q in 79%. However, the results of the latter two studies have to be interpreted with caution because the tumors under investigation were not exclusively Barrett cancers (see Table 1 and footnotes). Adenocarcinomas of the esophagogastric junction, which have been included in these studies, are known to share more similarities with gastric cancers, which are known to exhibit *c-myc* amplification quite frequently.

A polymerase chain reaction (PCR)–based investigation found *c-myc* amplifications in 43.6% (17 of 39 Barrett cancer cases under investigation) [7]. Although these results are more similar to the findings of our current investigation, this PCR approach is, again, inferior to CISH. A third study, in which overexpression of the c-myc oncoprotein was studied by Western blot analysis, revealed a high level of tumors overexpressing c-myc (18 of 20 cases; 90%). All these data

suggest that *c-myc* is an important molecular feature of invasive Barrett cancers, and especially low-level amplifications can frequently be found when a sensitive technique is applied.

c-myc amplifications have previously been shown to be less frequent or absent in precursor lesions (non-neoplastic Barrett esophagus and high-grade intraepithelial neoplasia) than in invasive Barrett cancer [7,8]. This might explain the lack of correlation of our results (obtained from invasive Barrett cancer specimens) with clinicopathological parameters such as pT/pN category, UICC stage (Table 2), and survival (Figure 2). Furthermore, this supports the concept that *c-myc* amplification is an early event during Barrett carcinogenesis, as indicated by previously published investigations [6–8].

The association of *c-myc* amplification with *VEGF-A* expression in the current investigation is well in accordance with the current understanding of the function of *c-myc*. Apart from various other effects by which *c-myc* contributes to carcinogenesis (i.e., promotion of cell growth, proliferation, loss of differentiation, and apoptosis) [12], the gene is also suspected to be involved in the regulation of angiogenesis [12,13]. c-myc deficiency is a lethal condition that has been shown to be due to the associated profound defects of vasculogenesis [13]. Embryos of *c-myc*$^{-/-}$ mice lack virtually the whole the vasculature. These defects can partially be addressed by the substitution of VEGF, suggesting that *c-myc* regulates angiogenesis through VEGF. This result has been substantiated by further studies indicating that Myc activation is a sufficient trigger to increase *VEGF* expression [12]. Our results in Barrett cancer (significant correlation of gene expression level with level of *c-myc* amplification) also support this link between *c-myc* and angiogenesis through the proangiogenic growth factor *VEGF-A*.

Due to a variety of implications in carcinogenesis, c-myc has been suggested as a promising target for molecular therapies [16]. Although these approaches are still in their infancy, promising strategies, including the application of antisense oligodeoxynucleotides, have been suggested to reduce *c-myc* expression. One other approach, specifically referring to the pathophysiology of Barrett carcinogenesis, relates to the reported modulation of *c-myc* expression by the contents of gastroesophageal refluxate. *In vitro* (cell culture) and *in vivo* (reflux models in animals) experiments have shown that bile acids activate c-myc [22]. Therefore, it was postulated that preventing esophageal epithelium from coming into contact with bile acids is one strategy against Barrett cancer [23]. Prevention of c-myc activation must be considered as one major aspect of such a strategy.

Conclusion

Our results of frequent (low-level and high-level) *c-myc gene* amplifications in a majority of Barrett cancers support the previously suggested importance of c-myc in this entity. *c-myc* amplification is likely to be an early event during Barrett carcinogenesis. Furthermore, our data suggest that an effect of the targeting of this gene is, at least in part, antiangiogenic in nature.

Acknowledgements

We thank Angelika Jahn for expert technical assistance in laboratory work, Reinhart Willers (University Düsseldorf) for statistical analyses, and Susanne Mach-Booms for maintaining our Barrett database.

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