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ORIGINAL ARTICLE

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MYEOV, a gene at 11q13, is coamplified with *CCND1*, but epigenetically inactivated in a subset of esophageal squamous cell carcinomas

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Abstract DNA amplifications at 11q13 are frequently observed in esophageal squamous cell carcinoma (ESC) and correlate with a malignant phenotype. Although this amplicon spans a region of several megabases and harbors numerous genes, *CCND1* and *EMS1* are thought to be the relevant candidates in ESC. We investigated whether the putative transforming gene *MYEOV*, mapping 360 kb centromeric to *CCND1* and activated concomitantly with *CCND1* in a subset of t(11;14)(q13;q32) positive multiple myeloma cell lines, represents a target of 11q13 amplification in ESC. To evaluate the role of *MYEOV* in ESC, we tested 31 ESC cell lines and 48 primary tumors for copy number levels of *MYEOV* and demonstrated that *MYEOV* was always coamplified with *CCND1*. However, *MYEOV* expression levels correlated only inconsistently with DNA amplification data. Treatment with the demethylating agent 5-aza-2′-deoxycytidine restored *MYEOV* expression in a subset of cell lines exhibiting DNA amplification without high *MYEOV* expression, suggesting that *MYEOV* is transcriptionally silenced by a DNA methylation mechanism in most of the latter cell lines. Our results indicate that *MYEOV* is a coamplified gene with *CCND1* at 11q13, but its activation is sometimes inhibited by an epigenetic mechanism.

Key words 11q13 Amplification · Esophageal squamous cell carcinoma · *MYEOV* · *CCND1* · *EMS1* · Epigenetic

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Introduction

It has been reported that amplification at 11q13 is involved in esophageal squamous cell carcinoma (ESC) as well as in several other types of solid tumors (Yoshida et al. 1993; Schuuring 1995; Schwab 1998). In addition, there is evidence that this amplification is associated with malignant phenotypes of ESC, such as invasiveness, metastasis, and poor prognosis (Yoshida et al. 1993; Adelaide et al. 1995; Shinozaki et al. 1996). Within the 11q13 amplicon, *CCND1* and *EMS1* are the only genes known to be both amplified and overexpressed, thereby making them target genes in ESC as well as other tumors exhibiting the 11q13 amplification (Schuuring 1995; Hui et al. 1997). However, this amplicon spans a 3–5 Mb region, and comprises numerous genes that have not been well characterized thus far (Map Viewer, http://www.ncbi.nlm.nih.gov/cgi-bin/Entretz/map-search).

The chromosomal band 11q13 is frequently involved in chromosomal translocations in various hematological malignancies (Mitelman et al. 1997), such as the translocation t(11;14)(q13;q32) in a subset of B-cell malignancies, especially in non-Hodgkin lymphomas and multiple myeloma (MM) (Mitelman et al. 1997; Ronchetti et al. 1999; Chesi et al. 2000). This type of recurrent chromosomal alteration juxtaposes *CCND1* to the IgH-5′ Eμ enhancer at 14q34.2, resulting in the deregulation of *CCND1* expression, which is considered to be a critical molecular event in the pathogenesis of t(11;14) hematological malignancies (Nakamura et al. 1997). Through application of the NIH/3T3 tumorigenicity assay, *MYEOV* was recently identified as a novel transforming gene and mapped at 11q13 (Janssen et al.

1999, 2000). Fine mapping of *MYEOV* by fluorescence in situ hybridization to extended DNA fibers showed that this gene was sublocalized 360 kb centromeric to *CCND1*. Interestingly, the breakpoints of MM cell lines with t(11;14) were localized within this 360-kb region between *MYEOV* and *CCND1*. Furthermore, in a subset of MM cell lines with t(11;14) and *CCND1* overexpression, overexpression of *MYEOV* was revealed as well, suggesting that *MYEOV* might act as a second oncogene or as a modifier of B-cell transformation (Janssen et al. 2000).

All these findings prompted us to examine the role of *MYEOV* in esophageal carcinogenesis. We therefore investigated the amplification and expression status of *MYEOV* together with *CCND1* and *EMS1* in our series of ESC cell lines as well as in primary tumors. Our results show *MYEOV* coamplification with *CCND1* in all cell lines and primary tumors tested, whereas *MYEOV* expression was only detected in a subset of cell lines carrying *MYEOV* amplification. Additional experiments with the demethylating agent 5-aza-2′-deoxycytidine (5-AzaCd) suggest that *MYEOV* transcription is epigenetically silenced by methylation within the 11q13 amplicon.

Materials and methods

Cell lines and primary tumors of ESC

All 31 human ESC cell lines (KYSE series) were established from surgically resected tumors (Shimada et al. 1992), and maintained in RPMI-1640 supplemented with 10% fetal calf serum. Data from comparative genomic hybridization analyses involving 29 of these lines have been reported elsewhere (Pimkhaokham et al. 2000). After obtaining written consent from each patient as prescribed by the respective ethics committees, ESC tumor samples were collected from 48 independent patients treated at the University Hospitals of Kyoto and Keio in the formed style as approved.

Southern, dot, and Northern blot hybridizations

For Southern blot analysis, 10-μg aliquots of *Eco*RI-digested DNA extracted from each cell line or normal peripheral blood cells were electrophoresed in 0.8% agarose gels and transferred to nylon membranes (BIODYNE B, Nihon Pall, Tokyo, Japan). For dot blot analysis, 2-μg of DNA from each primary tumor, each cell line, or normal lymphocytes was denatured with 0.4 N NaOH, and then transferred to nylon membranes (BIODYNE B, Nihon Pall). For Northern blot analyses, 10-μg samples of total RNA extracted from each cell line were size fractionated in 1.0% agarose/0.67 M formaldehyde gels and transferred to positively charged nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Tokyo, Japan).

Each membrane was hybridized with [α^{32}P]-deoxycytidine triphosphate-labeled *MYEOV* cDNA under appropriate conditions, washed, and then exposed as described elsewhere (Janssen et al. 2000; Imoto et al. 2001a, 2001b). Subsequently, membranes were hybridized with cDNA probes for *CCND1*, *EMS1*, and the human glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as an internal standard. Autoradiographic signals were analyzed using a BAS-2500 image analyzer (Fuji film, Tokyo, Japan). After the background was subtracted, an intensity of each signal was evaluated with normalization divided by *GAPDH* signal for each sample.

5-AzaCd treatment

To assess reactivation of *MYEOV* expression, we plated seven ESC cell lines (KYSE-30, 170, 200, 510, 790, 960, and 1170) in six-well tissue culture plates 24 h before treatment. Cells were treated with 10 μM of 5-AzaCd (Sigma Chemical, St. Louis, MO, USA) as described by Kusaba et al. (1999), and harvested after 7 days. Total RNA was extracted, electrophoresed, northern blotted, and analyzed as described earlier.

Results

Amplification of *MYEOV*, *CCND1*, and *EMS1* in ESC cell lines and primary tumors

As shown in Fig. 1A, 20 of 31 (64.5%) cell lines showed coamplification of *MYEOV*, *CCND1*, and *EMS1*. One cell line showed small amplification of *EMS1* alone (KYSE-30), and the remaining 10 cell lines (KYSE-70, 150, 170, 190, 270, 350, 450, 850, 1190, and 1440) showed no amplification of these genes. Because of a limited amount of DNA, we carried out dot blot analysis in primary ESC tumors, and identified that *MYEOV* and *CCND1* are always coamplified in 6 of 48 tumors (12.5%) (Fig. 2).

Differential expression pattern between *MYEOV* and *CCND1* in ESC cell lines

Northern blot analysis revealed that all 20 cell lines with amplification of *CCND1* showed its consequent overexpression. One cell line (KYSE-350) without *CCND1* amplification also showed high expression of this gene. Under the same exposure conditions (24 h), *MYEOV* expression was detected in the KYSE-1240 cell line only (data not shown). After longer exposure (14 days), we were able to detect marked *MYEOV* expression levels in nine cell lines (KYSE-170, 220, 410, 450, 1240, 1250, 1260, 2270, and 2650) and low to moderate *MYEOV* expression levels in 7 lines (KYSE-30, 150, 270, 350, 790, 850 and 2400) (Fig. 1B). Eleven lines (KYSE-110, 140, 180, 200, 510, 520, 590, 770, 890, 960, and 1170) exhibiting coamplification of *MYEOV* and *CCND1* did not show any *MYEOV* expression, although all of them showed elevated or high levels of *CCND1* expression (Fig. 1B). Seven cell lines showed *MYEOV* expression without concomitant *MYEOV* amplification (KYSE-30, 150, 170, 270, 350, 450, and 850).

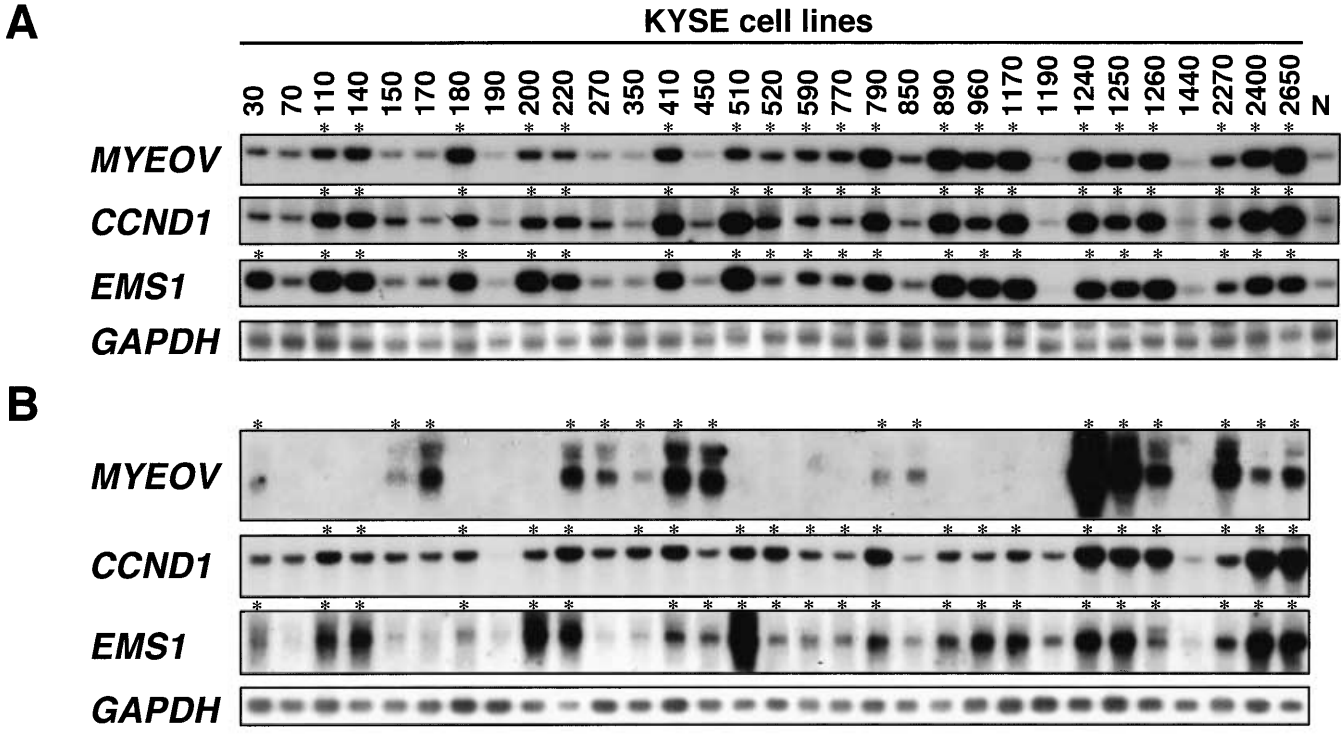

Fig. 1A,B. Southern and Northern blot analyses of *MYEOV*, *CCND1*, and *EMS1* in esophageal squamous cell carcinoma (ESC) cell lines. **A** Representative results of Southern blot analyses using *MYEOV*, *CCND1*, and *EMS1* probes and an internal control (*GAPDH*) probe in 31 ESC cell lines and normal peripheral lymphocytes from a healthy donor (*N*). *Asterisks* indicate amplification. **B** Representative results of Northern blot analyses using *MYEOV*, *CCND1*, and *EMS1* probes and an internal control (*GAPDH*) probe in the same panel of ESC cell lines. The exposure times for the *MYEOV*, *CCND1*, and *EMS1* probes were 14 days, 24 h, and 36 h, respectively. *Asterisks* indicate overexpression. Note the close correlation between DNA copy-number status and the expression level of *CCND1* and *EMS1*, but not *MYEOV*

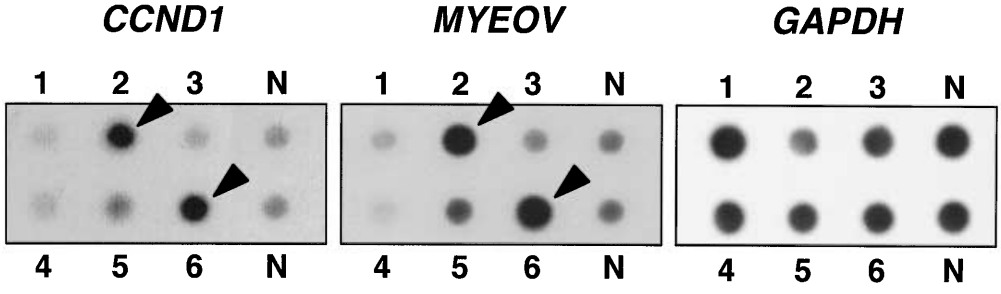

Fig. 2. Representative results of dot blot analysis of *MYEOV* and *CCND1* genes in primary ESC tumors. Cases 2 and 6 showed strong amplification signals with both *MYEOV* and *CCND1* (*arrowheads*). *GAPDH* served as a control probe. *N*, Normal control (peripheral blood leukocytes of a healthy donor)

Similar to *CCND1*, high expression of *EMS1* was consistently observed in cell lines with its amplification, although one cell line, KYSE-450, showed high expression of *EMS1* in the absence of its amplification.

Restoration of *MYEOV* expression after treatment with 5-AzaCd

A comparison between our Southern and Northern blot data revealed some discrepancies; for example, in some cell lines *MYEOV* amplification occurred without concomitant *MYEOV* expression. These data suggested that *MYEOV* expression might be regulated epigenetically; for example, by methylation. Therefore, we examined restoration of *MYEOV* expression in *MYEOV* amplification-positive/expression-negative ESC cell lines after 5-AzaCd treatment. Seven different cell lines (KYSE-30, 170, 200, 510, 790, 960, and 1170), of which one line (KYSE170) showed high *MYEOV* expression without concomitant DNA amplification, were treated with 5-AzaCd. Despite 5-AzaCd treatment, *MYEOV* expression levels in the KYSE-170 cell line were barely changed. On the other hand, *MYEOV* expression was clearly restored in most of the other cell lines (Fig. 3). Restored *MYEOV* expression levels were very prominent in three cell lines (KYSE-510, 790, and 1170), all of which showed distinct amplification of *MYEOV*, and their restored expression levels matched with their respective amplification levels (Fig. 3).

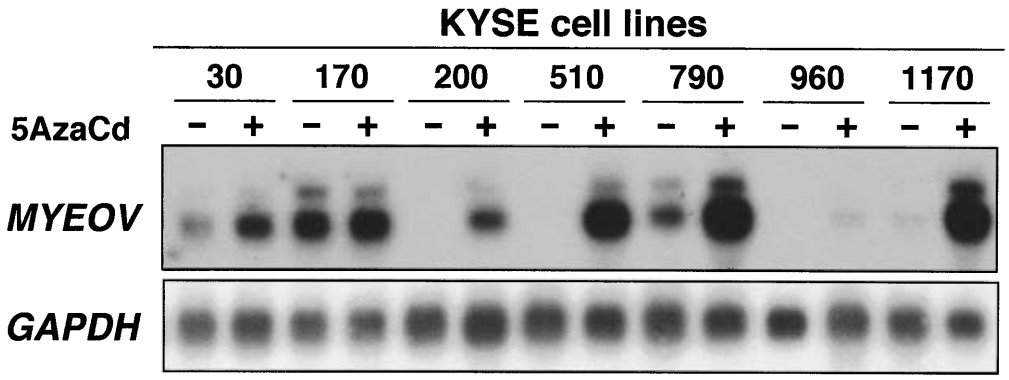

Fig. 3. Northern blot analysis of *MYEOV* in ESC cell lines treated with or without the DNA methylation inhibitor agent 5-aza-2′-deoxycytidine (5-AzaCd). Cells were treated with 10 μM of 5-AzaCd or vehicle for 7 days. *GAPDH* served as a control probe. Note the clear reconstitution of *MYEOV* expression after 5-AzaCd treatment in the ESC cell lines KYSE-510, 790, and 1170 at levels comparable to their relative DNA amplification levels. In KYSE-960, the *MYEOV* expression level was partially restored, but at a level incomparable to its amplification status. In KYSE-170 exhibiting high *MYEOV* expression without its amplification, *MYEOV* expression levels were not altered after 5-AzaCd treatment

Discussion

Amplification of the chromosomal region of 11q13 is commonly observed in various human cancers, including breast, bladder, ESC, and head and neck tumors (Knuutila et al. 1998). The amplicon involved has been reported to harbor several genes, including *CCND1*, *FGF4 (HSTF1)*, *FGF3 (INT2)*, and *EMS1*. Among them, the role of *CCND1* and *EMS1* in the formation of the amplification has been well established (Schuuring 1995). *CCND1* is located within 60 kb of *FGF4* (http://www.ncbi.nlm.nih.gov/cgi-bin/Entretz/map-search), whereas *EMS1* maps at least 800 kb telomeric to *CCND1* (Hui et al. 1997). Amplification data for *CCND1*, *FGF3*, and *EMS1* in a cohort of 747 breast cancers showed coamplification of *CCND1* and *FGF3*, but not of *EMS1* and *CCND1*, or *EMS1* and *FGF3* (Hui et al. 1997). In addition, *EMS1* amplification without concomitant *CCND1* and/or *FGF3* amplification was reported in a significant fraction (44%). Independent amplification of *CCND1* and *EMS1* has been reported in other types of tumors as well (Schuuring 1995; Meredith et al. 1995; Rodrigo et al. 2000). Consistent with those data, our results show that *EMS1* could also be found amplified solely, although it was coamplified with *CCND1* in most cases, whereas *MYEOV* was always coamplified with *CCND1* in cell lines and primary tumors of ESC. Taken together, our data are consistent with an amplification core spanning at least the region between *MYEOV* and *FGF3* in ESC. Several genes activated by DNA amplification in the 11q13 region may be located within this core region.

DNA methylation is thought to play a key role in the regulation of gene expression, and various genes show an inverse relationship between DNA methylation and transcription in both normal and malignant cells (Boyes and Bird 1992; Ponder 2001; Baylin et al. 2001). Our results showed prominent restoration of *MYEOV* expression after treatment with 5-AzaCd in three cell lines (KYSE-510, 790, and 1170), all of which exhibited distinct amplification of this gene, supporting the hypothesis that, despite its amplification, aberrant methylation of the *MYEOV* CpG islands inhibited its expression. Indeed, restored *MYEOV* expression levels were almost proportional to the level of *MYEOV* amplification in each of the cell lines after 5-AzaCd treatment. Because the genomic structure of the *MYEOV* gene, including its promoter region, has not yet been characterized, we could not evaluate the methylation status of the promoter region regulating *MYEOV* expression. Therefore, the possibility remains that the restored *MYEOV* expression by 5-AzaCd treatment may be induced indirectly via activating the expression of trans-acting factor(s) regulating *MYEOV* transcription. Another possible mechanism to modulate the transcription of the *MYEOV* gene independent of its genomic copy number would be by histone deacetylation (Struhl 1998). Histone deacetylases (HDACs) regulate the acetylation status of the core nucleosomal histones, which regulate the transcriptional activity of certain genes, and HDAC activity is generally associated with transcriptional repression (Struhl 1998). However, our preliminary study using Tricostatin A, a potent HDAC inhibitor, with or without 5-AzaCd, did not alter *MYEOV* expression, excluding this possibility (unpublished data). Further study will be necessary to determine the precise mechanisms for silencing *MYEOV* expression in tumor cells with its amplification.

Amplified chromosomal regions usually span a region of several megabases, consist of several amplification units, and harbor many different genes (Schuuring 1995; Schwab 1998). However, the number of target genes activated via amplification is usually limited (Imoto et al. 2001b; Schuuring et al. 1992; Yasui et al. 2001; Nakakuki et al. 2002; Platzer et al. 2002). Despite amplification, several genes in the amplicon are not upregulated, even though they are previously nominated candidate targets for amplification or known to be oncogenes (Platzer et al. 2002); the reasons have not yet been elucidated. Our results suggest that DNA methylation might be a mechanism for escaping upregulation of genes involved in amplification. Further

studies will be necessary to clarify whether DNA methylation plays an important role in gene silencing of different genes, both within the same amplicon and among different amplicons on different chromosomes.

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