MDM2 gene amplification in esophageal carcinoma

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Abstract. Esophageal cancer (EC) is one of the most common malignancies diagnosed in the Western world with an increasing incidence noted for esophageal adenocarcinoma (EAC). Despite improvements in staging, surgical procedures and postoperative treatments, the overall survival of patients with EC remains low. Murine double minute-2 (MDM2) acts as an oncogene by inducing the degradation of the tumor-suppressor protein TP53. In order to evaluate the *MDM2* gene amplification status in EAC and squamous cell carcinoma (SCC), we established a quantitative PCR (qPCR) assay, screening a total of 127 esophageal carcinoma cases for MDM2 amplification. Esophageal carcinoma cases with enhanced *MDM2* gene copy numbers were further analyzed by fluorescence *in situ* hybridisation (FISH) and MDM2 immunostaining. Among a total of 23 specimens (18%), identified by qPCR to possess elevated *MDM2* gene copy numbers, one third (6.3%) showed a cluster-like fluorescence pattern by FISH analyses and marked MDM2 protein immunostaining. MDM2 gene amplifications did not correlate with the occurrence of TP53 mutations. Due to the high therapeutic relevance of *MDM2* overexpression, but the high cost of FISH, we suggest a primary screening of *MDM2* copy number variations by qPCR, followed by detailed FISH analysis of the identified ECs.

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Abbreviations: AC, adenocarcinoma; CNV, copy number variation; EC, esophageal cancer; FFPE, formalin-fixed and paraffin-embedded; FISH, fluorescence *in situ* hybridisation; MDM2, murine double minute-2; qPCR, quantitative PCR; SCC, squamous cell carcinoma; SNP, single nucleotide polymorphism; TP53, tumor protein 53

Key words: esophageal cancer, MDM2 gene amplification, FISH, copy number evaluation, TP53 mutation status

Introduction

Esophageal cancer (EC) is the eighth most common cancer in the world; more than 450,000 people worldwide are affected. It has an extremely aggressive nature and poor survival rate with a 5-year overall survival rate of approximately 10-25%. The two main types are squamous cell carcinoma (SCC) and adenocarcinoma (AC). The incidence of esophageal adenocarcinoma (EAC) has risen during the last 20 years, mainly in the Western population. The single most important risk factor is Barrett's metaplasia. Esophageal SCC has the highest rates in areas of the East and Middle-East. The most important risk factors for SCC include smoking and alcohol intake. Most of the tumors are diagnosed in advanced stages and a cure can only be anticipated in patients with superficial cancers (1-4). Only about 30-40% of patients respond to chemotherapy. Several biomarkers with possible predictive power have been described. However, a pre-selection of patients with possible benefit of neoadjuvant treatment is not in clinical practice to date (5).

TP53 mutations are the most frequent mutations in ECs detected so far in several studies. Thus, between 40 and 50% of EC cases show a mutation of TP53. Hereby the prevalence of TP53 mutations in esophageal SCC might be slightly higher than that in EAC (6). However, although the high mutation rate of TP53 clearly demonstrates the crucial role of the tumor-suppressor TP53 in EC development, it has not yet led to individualised treatment or has not influenced prognosis (7-11).

The human homologue of the murine double minute-2 gene (*MDM2*) is a negative regulator of TP53. MDM2 is an E3 ubiquitin-protein ligase and its overexpression leads to TP53 ubiquitination and subsequent degradation linked to the loss of TP53 tumor-suppressor activity (12). Overexpression of *MDM2* may be due to gene amplification or to increased promoter activity of patients carrying the MDM promoter

T309G single nucleotide polymorphism (SNP) (13). Since many tumors have shown overexpression of MDM2, novel therapeutical approaches target MDM2 in order to restore TP53 tumor-suppressor activity (12-14).

In the present study, we investigated *MDM2* gene amplification by quantitiative PCR and fluorescence *in situ* hybridisation (FISH) in 127 ECs. Importantly, approximately 18% demonstrated moderately elevated *MDM2* copy numbers by qPCR, which may not affect overall *MDM2* overexpression in the tumor. However, 6.3% of all EC showed a strong enhancement of *MDM2* gene copies, that was linked to a cluster-like amplification pattern by FISH and to strong MDM2 immunostaining.

Materials and methods

Clinical and histological tumor evaluation. Esophagectomies were performed for patients with EC at the Department of Surgery, University of Cologne, Germany. Among 146 patients with locally advanced EC (cT3-4, cNx and M_0), tissue samples collected at the Institute for Pathology, University Hospital of Cologne were applied in our restrospective study. All specimens were used in accordance with the local policies of the Institutional Review Board of the University Hospital of Cologne. TNM staging was performed according to the criteria of the International Union Against Cancer (UICC) (17). Clinical staging consisted of endoscopy, endoscopic ultrasound, barium swallow, CT scanning of the abdomen and thorax and positron-emission tomography. Morphologic assessment of tumor regression was performed by an objective histopathologic examination as described previously (16). The resected specimens were fixed in formalin (10%). The whole tumor region including a safety margin was excised by a pathologist in a topographic order. Histological sections were prepared according to standard procedures of pathology.

FISH analysis. FISH was performed on interphase nuclei of 4-μm sections of formalin-fixed and paraffin-embedded (FFPE) tissue. Deparaffinised slides were subjected to several pretreatment steps. They were incubated in 0.2 M HCl for 20 min, followed by washing, and 80˚C heat pretreatment in pretreatment-solution (Abbott, Wiesbaden, Germany) for 30 min and further washing. Next, the tissue was digested for 1.5 h with protease, slides were washed again, and fixed in 4% buffered formalin. After washing, dehydration and air drying, an appropriate amount of fluorochrome-labeled probe mixture was applied to the tissue section. Sample DNA and probe mixture were co-denatured in a thermobrite hybridiser (Abbott) at 85˚C for 10 min, cooled down to 37˚C, and hybridised overnight in a humidified hybridisation chamber. After stringent washing and dehydration and air drying, the sections were counterstained using fluorescence mounting media containing (4',6-diamidino-2-phenylindole) (DAPI). FISH slides were evaluated with a fluorescence microscope (DM 5500; Leica Microsystems, Wetzlar, Germany) using appropriate filter sets. Orange (centromeric region) and green (target gene) signals were counted in 60 non-overlapping tumor cell nuclei from three tumor areas. Dual color FISH probes were derived from ZytoVision (Bremerhaven, Germany).

Table I. Sample cohort used in the present study (n=127).

	AC		SCC	
	Without neoadjuvant therapy	With neoadjuvant therapy	Without neoadjuvant therapy	With neoadjuvant therapy
Female (n)	7	6	6	4
Male (n)	38	36	11	19
Total (n)	45	42	17	23

AC, adenocarcinoma; SCC, squamous cell carcinoma.

Macrodissection and DNA extraction from the FFPE tissues. Six 10-μm sections of 127 FFPE esophageal tumor biopsies were used for macrodissection and subsequent DNA extraction as previously described (18). Briefly, for genomic DNA extraction, the lesional areas were marked on a haematoxylin and eosin (H&E) slide by a senior pathologist. After deparaffinisation, tumor tissue was macrodissected from the unstained slides and the tissue was lysed with proteinase K overnight. Subsequently, DNA purification was carried out with the BioRobot M48 robotic workstation and the corresponding MagAttract DNA Mini M48 kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

TP53 mutation analysis. The TP53 mutation status was evaluated by data analyses available from a comprehensive next generation sequencing approach which was performed on 68 ECs from a total of 127 tumors. Library construction on a hot spot cancer gene panel (Life Technologies, Darmstadt, Germany) and ultra-deep sequencing on the MiSeq platform (Illumina, San Diego, CA, USA) were performed according to Grünewald *et al* (19).

Copy number evaluation by real-time PCR. Evaluation of the MDM2 gene copy number variation (CNV) of the esophageal tumors was performed by quantitative PCR (qPCR). DNA from human embryonic kidney epithelial cells (HEK293) and DNA from 14 macrodissected, non-tumorous colon FPPE tissues were taken as non-*MDM2*-amplified reference DNA. Primer and probes for CNV assays of *MDM2*, *TERT* and *RNase P* were supplied by Applied Biosystems Life Technologies (Darmstadt, Germany).

Real-time PCR was carried out in triplicate, following the manufacturer's protocol. Copy numbers were then interpreted by the $\Delta\Delta$Ct method using the non-tumorous reference panel as calibrator and RNAse P as endogenous control. Evaluation of the RNAse P gene as the best endogenous control gene, demonstrating stable copy numbers of two alleles per cell in different reference panels (data not shown), was determined by standard curves and analysis of different non-amplified reference DNA.

Results

Efficient MDM2 copy number screening by qPCR. FISH analysis of gene amplification is laborious, costly and time-consuming. Therefore, we macrodissected the tumor areas from 146 EC

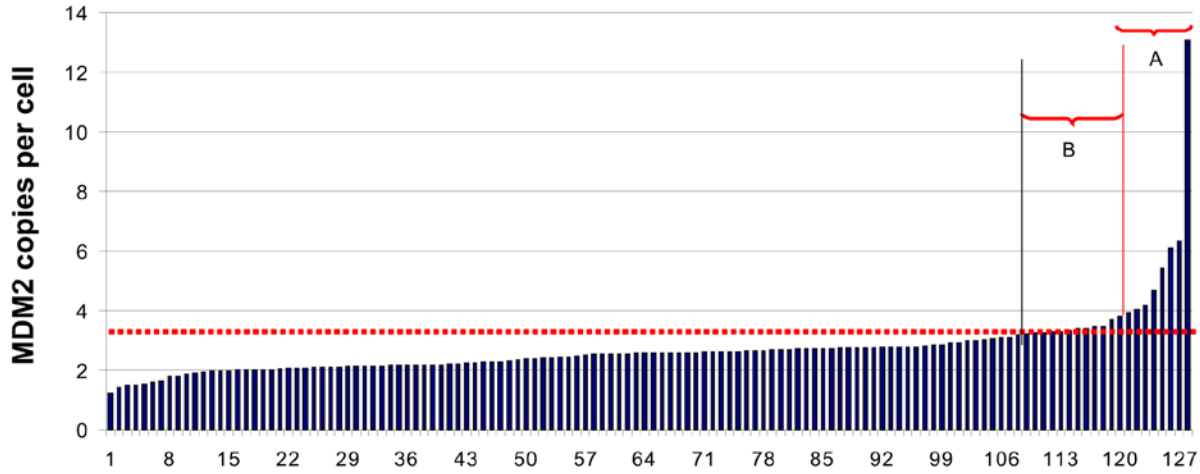

Figure 1. Copy number variation of the murine double minute-2 (*MDM2)* gene in esophageal carcinoma (n=127). Copy number variation (CNV) of the *MDM2* gene was analysed by real-time PCR using the RNase P gene for normalisation. Four or more copies per cell were interpreted as gene-amplified cases (A), whereas samples with <4 but >3 copies per cell were suggested to be putatively positve for *MDM2* gene amplification (B).

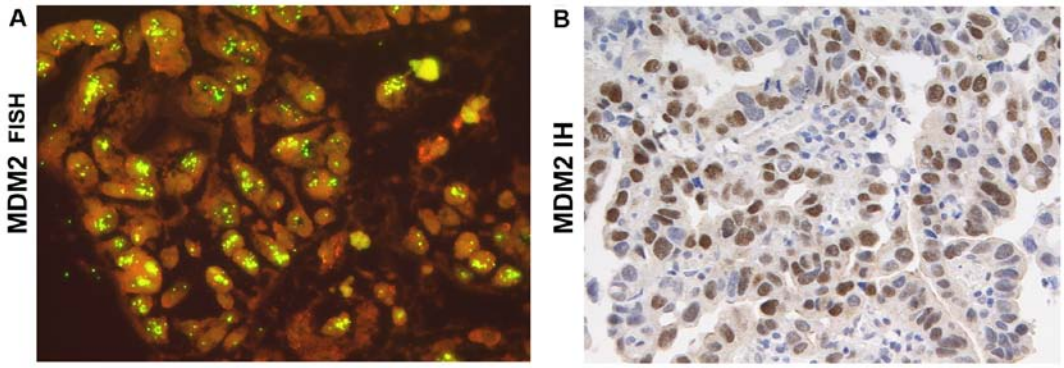

Figure 2. Murine double minute-2 (*MDM2)* gene amplification in esophageal carcinoma. (A) FISH demonstrated a cluster-like gene amplification of *MDM2*, which was linked to high MDM2 protein expression shown by (B) immunostaining (IH). FISH; fluorescence *in situ* hybridisation.

specimens and extracted DNA, in order to study *MDM2* CNVs by qPCR. DNA qualtity of 127 ECs (Table I) was sufficient to perform qPCR. Normalization of *MDM2* gene quantification was established, using RNAse P gene as a calibrator. Determination of RNAase P values in normal tissues ascertained normalisation to two copies per cell (data not shown).

Then, we screened the 127 ECs (Table I) for *MDM2* gene amplification. Fig. 1 demonstrates that eight EC (6.3%) samples showed an *MDM2* copy number of 4 and higher. These tumors were then studied by FISH showing tumor areas with high *MDM2* gene clusters (Fig. 2) and increased MDM2 protein expression in the nuclei of EC tumor cells.

Importantly, in some samples (n=15, 11.8%) we found mean values of more than three *MDM2* gene copies per cell by qPCR, indicating that these ECs might be also positve for *MDM2* gene amplification (shown in area B of Fig. 1). Therefore, we studied the tumor histology and the MDM2 protein expression of these ECs. Furthermore, gene amplification was analysed by FISH. In most cases, no or only very low gene amplification was shown by FISH and importantly, we did not observe the typical cluster-like amplification pattern of MDM2-positive tumor cells. In addition, ECs with slightly elevated *MDM2* copy numbers showed moderately enhanced MDM2 protein expression by immunohistology (Fig. 3).

However, a slightly increased *MDM2* copy number by PCR (shown in area B of Fig. 1) might also be due to low tumor cell content in the macrodissected EC area. Indeed, in some case with an MDM copy number >3 (Fig. 2B) only a few tumor cells were observed, that were scattered over the tumorous area, embedded by a high proportion of non-tumorous stroma cells. These cells clearly showed *MDM2* gene amplification by a cluster-like pattern of the *MDM2* FISH signals (Fig. 4).

MDM2 gene amplification in esophageal SCC and AC. From a total of 127 ECs, we identified by qPCR 8 tumors (6.3%) that carry highly elevated numbers of the *MDM2* gene (Table II and Fig. 2). There was no prevalence of *MDM2* gene amplification in AC or SCC (Table II).

Treatment with MDM2 inhibitors is a novel important therapeutical option, but depends on the TP53 wild-type form. Therefore, we searched the NGS data of the EC cohort and analysed whether tumors with *MDM2* amplification and overexpression harbor the mutated or wild-type TP53. From a total of 68 ECs, NGS data were available and revealed that 63% of the ECs had a pathogenic mutation in TP53. From the 8 samples, which were positive for *MDM2* amplification, 4 samples carried the wild-type and 4 were shown to carry the TP53 mutants.

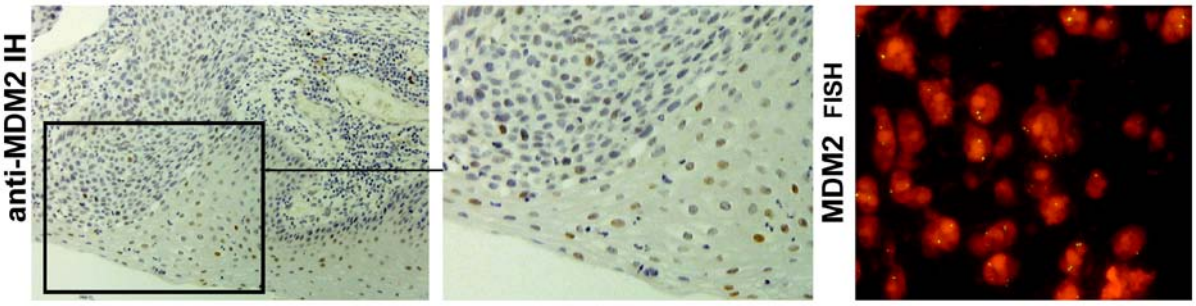

Figure 3. Murine double minute-2 (MDM2) expression in esophageal carcinoma with *MDM2*-borderline gene amplification. MDM2 immunostaining (IH) shows an increased, scattered expression pattern in a squamous esophageal carcinoma sample which showed marginally elevated *MDM2* gene copies by qPCR (CNV=3.3). In agreement with the low *MDM2* expression, no *MDM2* gene amplification was confirmed by FISH, although in some nuclei three *MDM2* copies were observed. FISH, fluorescence *in situ* hybridisation.

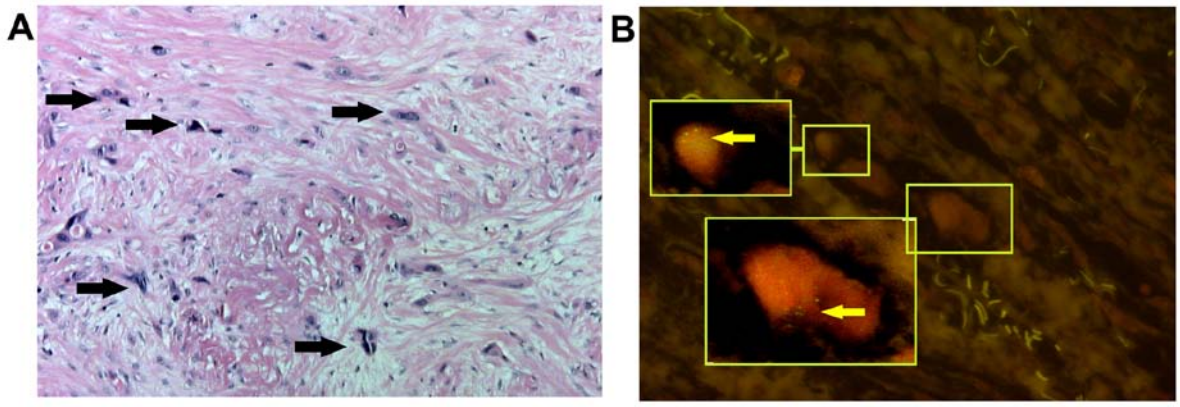

Figure 4. Low tumor cell proportion in several esophageal carcinoma samples. (A) Esophageal adenocarcinoma with a low number of tumor cells (arrows) scattered over the stroma with a high proportion of biomatrix fibers and myofibroblasts. H&E staining. (B) FISH demonstrated that some of the tumor cells, scattered over the stroma (yellow arrows), showed *MDM2* gene amplification by fluorescence signal clusters. FISH, fluorescence *in situ* hybridisation.

Table II. TP53 mutation status of the esophageal carcinoma samples with *MDM2* gene amplification.

Sample no.	Esophageal carcinoma	TP53 mutation status
1	SCC	p.C176W
2	SCC	p.R213[a]
3	AC	wt
4	AC	p.E286[a]
5	AC	p.R248Q
6	SCC	wt
7	AC	wt
8	AC	wt

[a]Stop codon. SCC, squamous cell carcinoma; AC, adenocarcinoma; wt, wild-type.

Discussion

MDM2 is an E3 ubiquitin ligase, which functions as a crucial oncogene by its interaction with TP53 leading to TP53 degradation. Due to its high impact on TP53 regulation, MDM2 is an important target of novel therapeutic strategies (12,14).

MDM2 overexpression was previously shown in many tumor types such as soft tissue sarcoma (20) and in non-small cell lung cancer (NSCLC) (21,22). In the present study, we investigated *MDM2* gene amplification in 127 EC specimens. Using qPCR assay, we identified 6.3% of the EC cases with a pronounced *MDM2* gene amplification, which were validated by a cluster-like signal pattern by FISH. In addition, the qPCR screening approach detected 15 borderline ECs with a moderate *MDM2* gene amplification. Although these ECs showed also no or only low *MDM2* gene alterations by FISH, in the ECs in which only a low number of tumor cells were recorded, high *MDM2* gene amplification was confirmed by the FISH assay. Since FISH is very laborious, costly and time consuming, qPCR is a highly efficient method by which to first screen tumors for *MDM2* gene amplification. The selected putative positives can then be further studied by MDM2 immunostaining and FISH. This economic approach is suggested to be of great importance for future EC treatment, in particular, since presently several small MDM2 inhibitors are in phase I of clinical trials, as summarised by Zhao *et al* (23).

There was no prevalence of *MDM2* gene amplification in the SCC or in AC cases. Taniere *et al* reported that *MDM2* amplifications were noted in SCC and AC in a small Asian cohort (n=23) with a frequency of 4% (24,25). Interestingly, the authors also described that the prevalence of *MDM2*

amplifications was much higher in Barrett's mucosa and adenocarcinoma of the cardia (25) reaching 19% of the cases.

In our study, the ECs with higher *MDM2* copy numbers showed also a pronounced staining for the MDM2 protein. Primary findings of Soslov *et al* demonstrated that >50% of AC cases showed *MDM2* overexpression. However, beside gene amplification, *MDM2* expression depends also on the T309G SNP which is associated with increased *MDM2* promoter activity (13,26,27,29). Furthermore, *MDM2* gene amplification might be due to an increased activity of the eukaryotic translation initiation factor 4E (eIF4E), shown to elevate *MDM2* expression in EC (28).

From a total of 127 patients, we studied the TP53 mutation status in 68 tumors demonstrating an overall TP53 mutation frequency of 63%. There was no significant difference in the TP53 mutation frequency in the *MDM2*-positive and -negative ECs. Thus, in summary, our findings on *MDM2* gene amplification and TP53 mutations revealed that therapeutic approaches with MDM2 inhibitors may be an important option for patients with EC independently in the case of the development of AC or SCC.

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