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Mdm2 mRNA expression in salivary gland tumour cell lines

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BACKGROUND: Murine double minute 2 (mdm2) is a cellular protooncogene, which, in conditions of overexpression or amplification, is capable of inactivating the functions of p53, leading to tumorigenesis. Immunoexpression of mdm2 in salivary gland tumours was previously found; however, it was necessary to find out if mdm2 was overexpressed. The aim of this study was to analyse the mRNA expression of mdm2 in salivary gland neoplasms and to correlate it to immunoexpression of p53 and p21 proteins.

METHODS: Specimens of different salivary gland neoplasms were obtained from surgical resections, and cell lineages derived from these tissues were established. RNA extraction was performed and mRNA expression was investigated using reverse transcription-polymerase chain reaction (RT-PCR). Cellular expression of p53 and p21 proteins was investigated by immunofluorescence technique.

RESULTS: Increased expression of mdm2 was found in the majority of cell lines analysed.

CONCLUSIONS: Comparing all results, we postulated that overexpression of mdm2 is related to the tumorigenesis and/or tumour progression of salivary gland neoplasms.

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Introduction

Murine double minute 2 (mdm2) is a cellular protooncogene, which, when overexpressed or amplified, is capable of inactivating p53, abolishing its antiproliferative function, thus leading to tumorigenesis (1). It was originally identified in a spontaneously transformed Balb/3T3 cell line (2) and, when amplified in rodent cells, it conferred high tumorigenic potential, suggesting its activity as an oncogene (3).

Amplification of mdm2 has been reported in soft tissue sarcomas (4, 5), and overexpression of mdm2 mRNA has been detected in breast carcinomas and breast carcinoma cell lines (6–8). It appears that in breast carcinomas and leukaemias, this occurs mainly by gain of function (without gene amplification) during transcription (9–12).

Previous studies from our laboratory demonstrated the presence of mdm2 in a set of minor salivary gland tumours using immunohistochemistry (13). The present study employed the multiplex reverse transcription-polymerase chain reaction (RT-PCR) to investigate mdm2 mRNA expression in a subset of human salivary gland cell lines. Additionally, because of the strong relationship between p53 and mdm2, the proteins p53 and its downstream p21 were analysed by immunofluorescence.

Materials and methods

Most cell lines were established by the explant technique and had been previously well characterised using immunofluorescence (data not shown), and HSG cell line was previously characterised by Shirasuna et al. (14). Presence of p21 and p53 was also assessed by this technique. The antibodies used were p21 (Calbiochem; clone AP1, 1 : 40) and p53 (Dako; clone DO-7, 1 : 50). Cell line names, origin and passage numbers are described in Table 1.

To analyse mdm2 mRNA expression, total RNA was extracted from confluent cell monolayers using TRIzol® (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instruction. As positive controls for mdm2 overexpression, RNA was extracted from K562 (ATCC, CCL243) cells, a leukaemia cell line, and from SjSA1, previously reported as OsA-CL (ATCC, CRL 2098) cells, an osteosarcoma cell line (4, 11).

Normal salivary gland RNAs (GN2 and GN3) were used as negative controls. Sections of 25 μm thickness from two normal salivary glands were minced in liquid nitrogen. The material was homogenised in TRIzol and RNA extraction

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Table 1 Cell lines names, passages, tumours and glands of origin; respective p53 and p21 immunoexpression and mdm2 mRNA (RT-PCR) expression

Cell line name (passage)	*Tumour of origin*	*Gland of origin*	*p53*	*p21*	*Mdm2*
M1 (p11)	Myoepithelioma	Palate	−	+	−
AP4 (p7)	Pleomorphic adenoma	Palate	−	+	+
AP5 (p4)	Pleomorphic adenoma	Submandibular	−	+	++
AP6 (p4)	Pleomorphic adenoma	Palate	−	+	++
CEM 2cl (p4)[a]	Epithelial myoepithelial carcinoma	Parotid	*	*	*
CEM epi (p6)[a]	Epithelial myoepithelial carcinoma	Parotid	+	−	−
CEM mio (p6)[a]	Epithelial myoepithelial carcinoma	Parotid	−	−	++
CAP (p2)[b]	Carcinoma ex pleomorphic adenoma	Submandibular	+	−	+++
CAPc (p6)[b]	Carcinoma ex pleomorphic adenoma	Submandibular	+	−	++
CAC2 (p10)	Adenoid cystic carcinoma	Parotid	+	−	+
CAC3 (p3)	Adenoid cystic carcinoma	Unknown	+	−	++
HSG	Adenocarcinoma (NOS)	Submandibular	+	−	−

+, Positive; −, negative.
+, Low increase in mdm2 expression.
++, Moderate increase in mdm2 expression.
+++, High increase in mdm2 expression.
−, No increase in mdm2 expression.
*, Results showed separately at CEM epi and CEM mio.
'a,b', originated from a single neoplasm, respectively.

was carried out following the manufacturer's instructions. Purity of all RNA was determined by UV spectrometry.

Total RNA was subjected to first-strand reverse transcription using random primers for synthesising cDNA (Life Technologies, Gaithesburg, MD, USA).

Complementary DNA (cDNA) created was then subjected to PCR to amplify the genes for mdm2 and β-actin, a housekeeping gene. Each PCR reaction contained 5 μl cDNA, 15 pmol of primer, 10 mM dNTP, 2.5 μl 10 × PCR buffer, 5 U TAQ polymerase (Gibco BRL, Life Technologies), and the volume was made up to 25 μl miliQ autoclaved H_2O. Primers used were mdm2 sense 5′-TGAAGGTTTCTCTTCCTGAAG and mdm2 antisense 3′-TTATTAAAGTCTGTTGGTGCA; β-actin sense 5′-CCTTCCTGGGCATGGAGTCTTG and β-actin antisense 3′-GGAGCAATGATCTTGATCTTC. PCR-amplified products were 335 and 220 bp in size, respectively.

Samples were amplified through 28 consecutive cycles to avoid the exponential phase of both primers (previously tested and established). Each amplification cycle consisted of a denaturation step at 94°C for 1 min, primer annealing at 57°C for 1 min and extension at 74°C for 4 min.

PCR products were electrophoresed through 2% agarose gel and visualised using ethydium bromide under UV light. The image was analysed by scanning densitometry (EDAS-Kodak), and band intensities were converted to numerical data. The mdm2:β-actin ratio was established and was then transformed to percentage values, and the mean values were assessed.

Results

Cells derived from the three pleomorphic adenomas used in this study (AP4, AP5 and AP6) were large and spindle-like. Cells that were precedents from myoepithelioma were polyedral and spindle-like. All these cells were p21-positive.

Carcinoma ex-pleomorphic adenoma-derived cells from various passages were studied. Cultures bearing premature passage numbers (CAP) showed two cell types – epithelioid small cells and large cobblestone cells (the latter was frequently seen in histological sections of the neoplasia). At passage number 6 (CAPc), only the small epithelioid cells remained. Both cell types were p53-positive.

Adenoid cystic carcinoma-derived cells (CAC2 and CAC3) were mainly polyhedral with elongated projections. These cells were p53-positive. Adenocarcinoma-derived cells (HSG) were epithelioid in shape, being positive for p53, as described previously in the literature (14).

The epithelial–myoepithelial carcinoma cell line was composed of two cell types – a spindle elongated cell (CEM mio), negative for both p53 and p21, and a cobblestone epithelioid cell (CEM epi), which was p53-positive. Cells from this line, from different passages, were analysed further. Early passages (CEM 2cl) demonstrated the presence of both cell types, and further passages (P6) showed each cell type separately. The imunofluoresecence results are shown in Table 1 and Fig. 1.

The criteria used to determine mdm2 mRNA overexpression was established by Bueso-Ramos et al. (11), by considering the relative ratio between mdm2 and β-actin; a ratio between 20 and 40% is considered a small increase, 41–80% a moderate increase and greater than 80% a high increase. Mdm2 mRNA expression seen in negative controls (GN2 and GN3) was less than 10%. Positive controls, represented by K562 and SjSA1, showed moderate and high increase in the expression of mdm2 mRNA levels, respectively.

Most cell lines analysed showed increased expression of mdm2. Small increases were found in cell lines derived from pleomorphic adenoma (AP4), epithelial–myoepithelial carcinoma (CEM 2cl), carcinoma ex-pleomorphic adenoma (CAPc) or adenoid cystic carcinoma (CAC2); moderate increases were seen in cells derived from pleomorphic adenoma (AP5 and AP6), epithelial–myoepithelial carcinoma (CEM mio) or adenoid cystic carcinoma (CAC3); high increases were observed in cells derived from carcinoma ex-pleomorphic adenoma (CAP) and no mdm2 mRNA increase was found in cell lines derived from myoepitelioma (M1), epithelial–myoepithelial carcinoma (CEM epi) and adenocarcinoma (HSG). All the results described are depicted in Figs. 1–3 and Table 1.

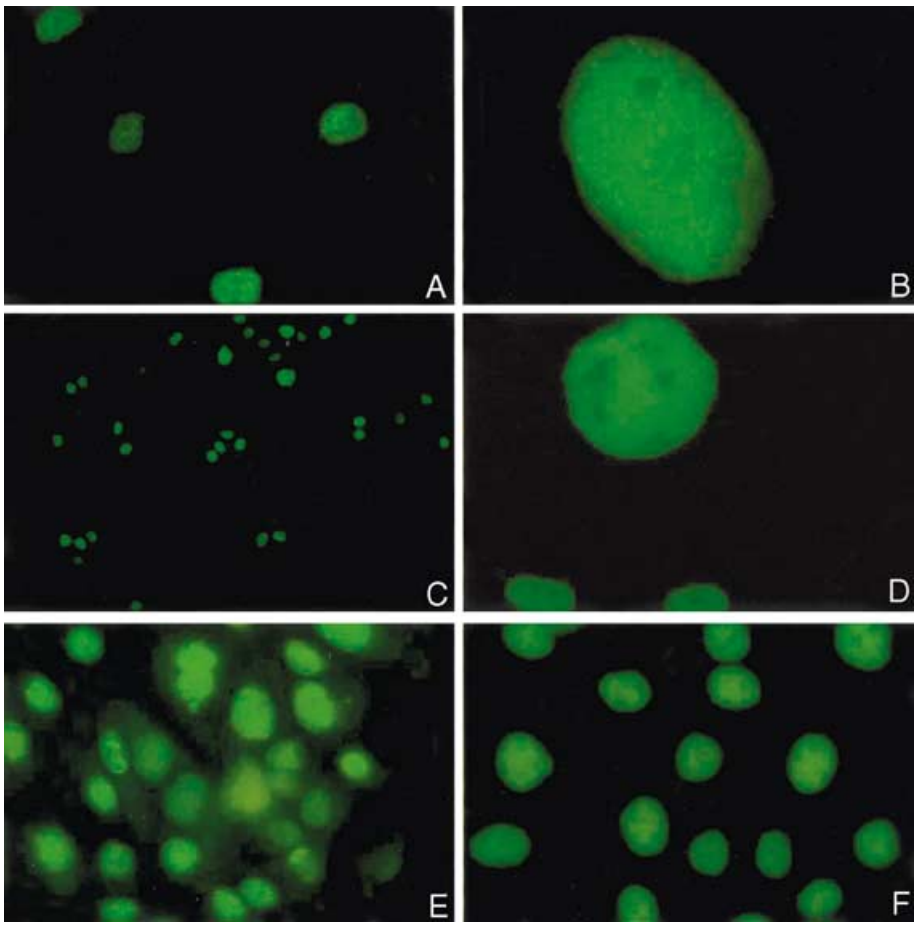

Figure 1 Immunoexpression of p21 and p53 in the salivary gland tumour cell lines. A and B – Nuclear positivity for p21 protein in AP6 cells (lower and greater view); C and D – Nuclear positivity for p53 protein in CAC2 cells (lower and greater view); E – Nuclear positivity for p53 protein in CEM2cl cells (the positivity is seen only in the epithelial subtype); F – Nuclear positivity for p53 protein in HSG cells.

Discussion

The results presented here clearly demonstrate that there is an overexpression of mdm2 in most salivary gland tumours analysed.

Alterations of mdm2 are mainly because of overexpression and amplification and the latter is commonly described in mesenchyme-derived tumours (4, 5). Overexpression may occur by mechanisms other than gene amplification. Gain of function during transcription has been described in breast carcinomas and leukaemias (9–12).

In salivary gland tumours, only one cytogenetic study has shown the presence of double-minutes in a case of pleomorphic adenoma (15); however, these results were not related to mdm2 gene alterations as mdm2 had not been well described by that moment. A previous immunohistochemical study performed in our laboratory demonstrated the expression of mdm2 in most salivary gland tumours analysed (13). With these studies in mind, the aim of the present study was to analyse mdm2 mRNA overexpression.

In addition to amplification and gain-of-transcription function, overexpression of mdm2 could be related by the release of mdm2 inhibition by $p19^{ARF}$ protein as described

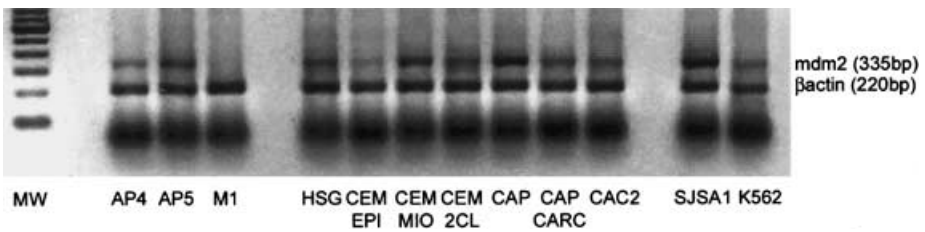

Figure 2 mRNA expression of mdm2 and β-actin in benign and malignant salivary gland neoplasms and positive controls. AP4, AP5 and M1 represent the benign tumours; HSG, CEM epi, CEM mio, CEM 2 cl, CAP, CAP carc and CAC2 represent the malignant tumours and SJSA1 and K562 represent the positive controls. Note that the expression of mdm2 varies depending on the tumour and the expression of β-actin, which is the internal control, is constant.

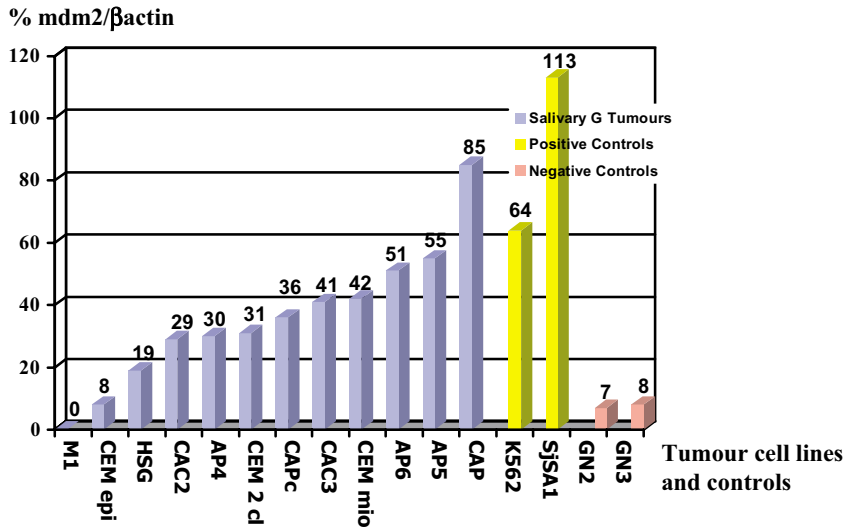

Figure 3 Mdm2/β-actin medium ratio in salivary gland tumour cell lines, positive and negative controls. 0–20%: No increase in mdm2 expression; 21–40%: Low mdm2 overexpression; 41–80%: Moderate mdm2 overexpression; 81+: High mdm2 overexpression.

by Pfister et al. (16) in bladder carcinoma. In normal conditions, $p19^{ARF}$ acts by attenuating mdm2-mediated degradation of p53, thereby stabilising p53 (17). The $p19^{ARF}$ is located at 9p21, and in salivary gland neoplasms, LOH has been described exactly in this locus (18). The mdm2 alterations found in our study may therefore represent transcriptional gain of function associated to defects in degradation of mdm2 products.

Overexpression of mdm2 was not observed in HSG cells, and p53 nuclear accumulation was demonstrated by immunofluorescense. These results are in agreement with the mutated p53 observed as an event in tumorigenesis in this cell line that was established after irradiation of a human salivary gland (14). Together, these data suggest that nuclear accumulation of p53 in HSG cell line may occur, in part, because of lack of mdm2 activation by a mutant p53 protein as described in cell lines analysed by Chen et al. (19). Therefore, the role of mdm2 in tumorigenesis and/or tumour progression in HSG cell line is probably absent.

A different mechanism seems to occur in AP4, AP5, AP6 (from pleomorphic adenoma), CAP and CAPc (from carcinoma ex-pleomorphic adenoma) cell lines. In pleomorphic adenoma, variable overexpression of mdm2 was found – moderate increase in AP5 and AP6 and small increase in AP4 cells. Additionally, p21 immunoexpression was observed without accumulation of p53, which would indicate a wild-type p53 protein. These sets of results indicate that mdm2 alteration could be an early event in the neoplastic development of pleomorphic adenoma. This suggestion may be confirmed by the results seen in carcinoma ex-pleomorphic adenoma (malignant transformation of pleomorphic adenoma), in which high increase of mdm2 mRNA expression was found in CAP and low increase was found in CAPc. Both cells lines showed nuclear accumulation of p53, and this finding is according to the literature considering that p53 gene mutation may be responsible for most cases of malignant transformation of pleomorphic adenomas (20). Therefore, mdm2 accumulation presumably progress from the benign neoplasms through malignant transformation, insinuating that this accumulation may increase cellular instability and, hence, favour p53 mutation. In other words, from an early event related to the development of benign tumours, mdm2 gene seems to play a part in tumour progression in salivary gland neoplasms. The low increases of mdm2 expression observed in CAPc may be related to loss of tissue induction as this cell line was studied in a later passage than CAP cell line.

Alterations of p53 in salivary gland tumours have been conflicting. In adenoid cystic carcinoma, p53 has been linked to tumorigenesis and tumour progression, being mutated in 100% of high-grade tumours (solid subtype; 21, 22). Our results indicate that accumulation of mdm2 is probably linked to tumour progression because in CAC2 derived from a less aggressive tumour subtype (cribriform), low overexpression was observed, whereas CAC3 obtained from a more aggressive tumour subtype (solid) showed moderate overexpression. There is always the possibility that the alterations found in p53 have occurred in a lower grade stage of the tumour. Both presented nuclear accumulation of p53 and negativity to p21, indicating an altered p53 protein. Alternatively, the differences in mdm2 expression between these two cell lines could be explained by their analysis in different passage numbers. CAC3 cells were closer to the explant and this fact may have been determinant in its higher mdm2 expression probably because of reminiscent signalling of tissue induction.

Epithelial–myoepithelial carcinoma is a bi-phasic tumour, composed of two cell types – epithelial and myoepithelial. In less aggressive neoplasms (tubular pattern), both cell types are present; however, during tumour progression, myoepithelial cells predominate, while epithelial cells almost disappear. Moderate mdm2 increases were observed in myoepithelial cells derived from this tumour (CEM mio). In contrast, there was no increase in mdm2 expression in epithelial cells (CEM epi); p53 nuclear accumulation was only detected in the epithelial cells. The predominance of

myoepithelial cells indicates a higher proliferative capacity of this cell, which may be, in part, explained by its mdm2 expression.

Accumulation of nuclear p53 was only seen in epithelial cells in epithelial–myoepithelial carcinoma; however, the absence of p53 in myoepithelial cells cannot be interpreted as presence of wild-type protein as expression of p21 was not detected in this cell type. The literature suggests that mdm2 can lead to degradation of p53 (23–25). Only a small quantity of mdm2 is necessary to degrade large amounts of p53, blocking its activity. This occurs even when stable mdm2/p53 complexes are formed but not detected (23). This phenomenon could be occurring in the myoepithelial cells of epithelial–myoepithelial carcinoma. In contrast, in epithelial cells of the epithelial–myoepithelial carcinoma, the nuclear accumulation of p53, interpreted as altered protein, could not be explained by complex formation, as there was no increase in mdm2 expression. Similar to the mechanism observed in HSG cells, the nuclear accumulation of p53 in the epithelial cell may be, in part, because of failure in mdm2 transcription by defective p53 activation, leading to a lack of complex formation and, consequently, failure of p53 degradation. These pieces of evidence suggest that either distinct tumorigenic stimulus may be present in epithelial–myoepithelial carcinoma, or that the same stimuli may result in different cell responses, depending on the cell type.

The cell line derived from a myoepithelioma showed p21 immunoexpression and no p53 positivity, suggesting a wild-type p53 protein. The mRNA analysis showed the lowest mdm2 expression. These results deserve further investigation as the passage analysed was far from tumour explant and mdm2 tissue induction could be lost as described in other cell lines.

Expression of p53 and p21 proteins varied according to each cell line studied. In cell lines derived from more aggressive neoplasms – adenoid cystic carcinoma, carcinoma ex-pleomorphic adenoma and adenocarcinoma – there was nuclear accumulation of p53 but no detection of p21. In cell lines derived from benign tumours, p21 expression was observed. However, relationship between these data and tumour progression was not possible because of lack of clinical information.

The presence of p21 is not only related to the apoptotic process via p53 (26), as its expression can also be found during cellular differentiation or senescence (27). Therefore, detection of p21 in benign tumours may also be explained by these alternative functions. This fact needs to be considered in the analysis of pleomorphic adenoma cells as this tumour presents areas of ductal formation with mucus production, chondroid areas and squamous metaplasia.

Overexpression of mdm2 was present in the majority of cell lines used in this study and is probably related to the tumorigenesis and/or tumour progression of at least a set of the tumours analysed. In cells derived from benign tumours, p53 expression was absent. In cells derived from malignant tumours, nuclear accumulation of p53 evidenced by immunofluorescence was variable, depending on the cell phenotype. The role of mdm2 in tumorigenesis is also related to other mechanisms such as interactions with TGFβ, E2F1 and Rb (28–30), and these mechanisms are currently under investigation in our laboratory. Further studies on the role of mdm2 in salivary gland neoplasms may be carried out in order to elucidate their pathophysiological behaviour and consequently improve the treatment available to patients in future.

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