# The prognostic value of MDM2 overexpresion in superficial urothelial bladder carcinoma

Lúcio Santos[1], Marta Koch[2], Céu Costa[2], Teresina Amaro[3], Catarina Lameiras[2], Sofia Pereira[2], Paula Lopes[4], Begoña Criado[5], Alberto Koch[6], Carlos Lopes[7]

1 - Departamento de Oncologia Cirúrgica – Assistente Hospitalar de Cirurgia
2 - Unidade de Cirurgia Experimental e Investigação de Tradução (Grupo de Investigação do Cancro da Bexiga) – Licenciados em Biologia
3 - Serviço Anatomia Patológica – Assistente Graduada
4 - Serviço Anatomia Patológica – Técnica de Anatomia Patológica
5 - Unidade de Cirurgia Experimental e Investigação de Tradução(Grupo de Investigação do Cancro da Bexiga) – Prof. Doutora e Investigadora
6 - Serviço de Urologia – Assistente Graduado
7 - Serviço Anatomia Patológica – Prof. Doutor e Chefe do Serviço

Instituto Português de Oncologia – Porto, Portugal
Rua Dr. António Bernardino de Almeida, 4200-072, PORTO, Portugal – Fax +351 22 502 6489
e-mail: ucib@ipoporto.min-saude.pt

## Resumo

O significado prognóstico da acumulação da p53 e a sobre-expressão do MDM2 nos carcinomas uroteliais superficiais e de morfologia papilar da bexiga (CUB) não está ainda estabelecida. Assim as proteínas p53 e MDM2 foram estudadas em função do grau de diferenciação e de invasão da lamina própria, com o objectivo de esclarecer o seu significado prognóstico.

**Métodos**

Em Trinta e cinco CUBs foi estudada a imunorreactividade para a p53 e MDM2 usando para tal anticorpos monoclonais específicos (DO7 e IB10).

**Resultados**

Sobre-expressão da MDM2 foi observada em 51,4% dos casos e correlacionou-se significativamente com a taxa de recidiva e com a sobrevivência livre de recidiva. A acumulação da p53 não se associou com o prognóstico. Estes marcadores não se associaram com o grau de diferenciação. Dos casos MDM2 positivos 66,7% eram pTa no entanto, nestes casos, a acumulação da p53 foi observada em apenas 33,3% destes. Aparentemente o MDM2 não se relaciona com a invasão da muscular da mucosa.

**Conclusão**

O MDM2 revelou-se neste estudo um factor de prognóstico nos carcinomas superficiais da bexiga.

## Abstract

**Purpose**

p53 accumulation and MDM2 overexpression prognostic significance in superficial and papillary Urothelial Cell Carcinoma (UCC) of the bladder is not established yet. Therefore, MDM2 and p53 protein were investigated focusing the grade and lamina propria invasion in order to identify their prognostic significance

**Methods**

Thirty-five archival UCC were studied by immunohistochemistry using monoclonal antibodies DO7 against p53 and IF2 against MDM2.

**Results**

MDM2 overexpression was observed in 51.4% of the cases and correlated with recurrence rate and recurrence-free survival. The p53 accumulation, was not related to prognosis. These markers have not significantly distinguished the high grade tumours from low grade superficial ones. The MDM2 positive cases were more frequent in pTa cases (66.7%) contrasting with its decreased frequency of p53 accumulation (33.3%). Apparently overexpression of MDM2 is not related with muscular mucosae invasion.

**Conclusions**

The MDM2 status appears to be a prognostic marker in superficial urothelial bladder carcinomas.


**Key Words:** MDM2, p53, Bladder cancer, prognosis

## Introduction

Bladder cancer is one of the most frequent tumour, approximately 80% are limited to the mucosa and lamina propria at presentation and are usually called superficial tumour (1). Recurrence rates are high (30%-80%) and 10 to 30% will subsequently progress (2). For this reason Murphy defends that the term superficial bladder cancer should be abandoned (3). He argues that 50% of these tumours are non-aggressive neoplasms but the remainder, despite superficial morphology, truly have an aggressive behaviour illustrated by invasion of lamina propria at the time of initial detection, high rate of recurrence and/or progression (3). This last subgroup is also associated with a high cancer-related death rate (4).

The substaging of T1 cases based on the depth of invasion can predict cancer progression. Unfortunately, the evaluation of muscularis mucosae invasion is often very difficult (5).Molecular tests could achieve important improvements in the identification of these tumours if they could distinguish the aggressive ones. Alteration of the TP53 suppressor gene has been reported in advanced urothelial carcinomas, carcinoma in situ (Tis) and urothelial dysplasia. Despite the correlation between p53 nuclear accumulation and progression in Tis and invasive tumours, its prognostic significance in superficial papillary bladder cancer is not established yet (6). Benardini et al. studied the predictive value of muscularis mucosae invasion and p53 nuclear accumulation in function of the progression of stage T1 bladder carcinoma. They showed in univariate analysis, a significantly association between p53 expression and tumour progression, however only the muscularis mucosae invasion was a predictor of progression in multivariate analysis (7). The MDM2 proto-oncogene overexpression has been shown to produce a malignant phenotype in 3T3 fibroblasts (8). MDM2 regulates the transcriptional function of p53. Under physiological conditions MDM2 expression is upregulated by wild type p53. Conversely, p53 will be complexed and thereby inactivated or targeted to degradation by MDM2 (9). MDM2 amplification has been found in soft-tissue sarcomas and other malignant tumours (10-11). In bladder cancer, high levels of MDM2 and p53 proteins have been described, in approximately 18% of this tumours both proteins are highly expressed (12-13). Lianes and Barbareschi M. et al. studied the MDM2 and p53 immunoreactivity in bladder cancer specimens and have observed no correlation between p53 nuclear accumulation and MDM2 overexpression (12-13). Overexpression of MDM2 was reported in a high proportion of superficial tumours and at lower frequency in high stage and grade tumours (14). As far as we are concerned the relationship between lamina propria invasion and MDM2 status, was not studied yet, then, in this study, p53 nuclear accumulation and MDM2 overexpression were examined in superficial and papillary Urothelial Cell Carcinoma (UCC) of the bladder focusing the grade and lamina propria invasion in order to identify the clinical significance of these alterations and clarify the prognosis of these patients.

## Material and Methods

### Patients, specimens and definitions

Thirty-five tumour specimens were obtained between December of 1989 and December of 1996 from 15 females and 20 males with UCC (table2) who underwent surgery (trans-urethral resection), at Department of Urology at Instituto Português de Oncologia do Porto.

Recurrence was defined as the, histologically – proven, reappearance of the tumour after curative treatment. Progression was defined as the increase of stage and/or grade in tumour recurrence. The recurrence-free survivals were defined as the intervals between the date of surgical treatment and the date of the clinical event or the last clinical assessment without recurrence.

### Pathological classifications

Histological typing and grading of UCC was performed according to the consensus (15). The tumor stage was determined according to the UICC classification modified by WHO/ISUP recommendations (16). Thus, tumour with invasion of the connective tissue superficial to the level of muscularis mucosae were classified as T1a and those with invasion of the muscularis mucosae were classified as T1b.

### Immunohistochemistry

Paraffin sections were immunohistochemically analysed for p53 and MDM2 expression using a standard avidin-biotin technique.

Briefly, 4.5 μm sections were deparaffinized with xylene and ethanol, submitted to microwave retrieval treatment and endogenous peroxidases were blocked using H2O2 in methanol for 20 min.. Nonspecific protein binding was blocked with 10% fetal bovine serum in phosphate-buffered saline (PBS). For p53 immunostaining, the primary antibody (DO-7, Dako®) was applied in a 1:50 dilution in BSA overnight at 4ºc. Concerning MDM2, the primary antibody (IB10, Novocastra®) was applied in a 1:50 dilution in BSA overnight at 4ºc. After washing in PBS, the sections were incubated the secondary antibody (biotinylated rabbit anti-mouse immunoglobulins) using a K335 *Dako*® kit, at room temperature for 10 min. Sections were washed again and reaction products were visualized with 0.05M Tris/HCl, 3,3' – diaminobenzidine tetrahydrochloride 0.05% (DAB) and hydrogen peroxide 0.01%, as chromogen. Finally, the sections were counterstained with hematoxylin for 5 min.

Appropriate positive controls were used for each antibody, and negative controls consisted of the replacement of the primary antibody for 2.5% BSA in Tris-buffered-saline (TBS).

### Immunohistochemical scoring

Positive staining was defined as the presence of ≥ 20% antibody-stained nuclei (p53 and MDM2) as reported by others groups (12,14,17). The entire section was screened to find the region with maximum fraction of positively and contiguous stained nuclei (p53 nuclear accumulation and MDM2 overexpression). The percentage of positively stained nuclei was scored in this region using the 40x objective. A total of 200 malignant cells were counted in each tumour. Immunohistochemical evaluation was done by two independent observers (T.A. and L.S.).

### Statistical analysis

Chi-square and when appropriate, Fisher's exact test, were used to compare categorical variables (univariate analysis). Recurrence-free survival rates were calculated by the method of Kaplan-Meier and the log-rank test was used to compare curves for 2 or more groups. We assessed the prognostic significance of the immunoreactivity for the factors in study by univariate analysis. Cox regression models were used to calculate crude OR (Odds ratio) and their 95% intervals associated with disease recurrence for clinicopathological parameters (stage, grade and multifocality) and biopathological markers with abnormal expression (p53 and MDM2). All statistical analyses were run on SPSS 8.0 software (SPSS Inc.). A p value less than 0.05 was accepted as statistically significant.

## Results

Among the 35 cases studied, 9 tumours were confined to the bladder mucosa (pTa), 26 showed invasion of the lamina propria (22 cases pT1a, 4 cases pT1b). There was a significant association ($p<0.01$) between tumour stage and tumour grade: 6 (66.7%) of the 9 pTa tumours were low grade. From the 22 pT1a UCCs, 18 (81.8%) were high grade and all (100%) pT1b. Multifocality was observed in 6 (19.4%) cases. Eighteen of the 35 cases (51.4%) experienced tumour recurrence and 4 cases (22,2%) tumour progression during a median follow-up time of 80.7 months (min – 5 months, max – 122.1 months).

The immunoreactivity for p53 and MDM2 was not significantly associated with tumour grade and stage (Table1). When analysing the immunoreactivity

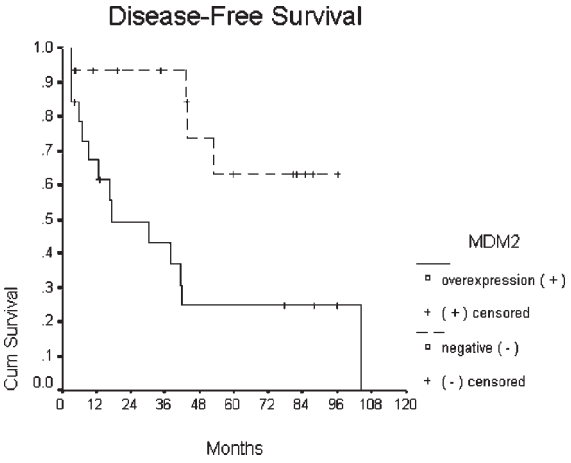


Fig. 1 – Kaplan-Meier survival curve demonstrating the relationship between Disease-Free Survival and MDM2 status. (Log rank – p< 0,01)

for p53 in the recurrences we observe that all specimens were p53 positive.

The univariate analysis revealed that only MDM2 overexpression was significantly related to tumour recurrence (P<0.02) (Table 2). Cases with MDM2 overexpression had a significantly lower recurrence-free survival (Fig 1). These survival differences were maintained when performing survival analysis by each stratum of all the variables studied. All cases with progression had MDM2 overexpressed (3 cases with phenotype MDM2+ /p53 – and 1 case with MDM2+/ p53+). The multivariate analysis showed that MDM2 overexpression was an independent prognostic factor (p= 0,01). The recurrence rate for MDM2 overexpression cases was 7.09-fold higher than for the cases without MDM2 overexpression (Table 3).

Table 1 – Correlation between p53 accumulation, MDM2 overexpression and tumour stage and grade

| Stage | Nº cases | p53 accumulation | MDM2 overexpression |
|---|---|---|---|
| Ta | 9 | 3 (33.3%) | 6 (66.7%) |
| T1a | 22 | 12 (54.5%) | 11 (50.0%) |
| T1b | 4 | 2 (50.0%) | 2 (50.0%) |
| Grade | | | |
| Low grade | 10 | 3 (30.0%) | 5 (50.0%) |
| High grade | 25 | 14 (56.0%) | 14 (56.0%) |

No statistically differences were observed

Table 2 – Correlation between tumour recurrence in 35 patients with superficial bladder cancer and clinicopathological and molecular parameters.

| Variables | N | With Recurrence (n = 18) | Without Recurrence (n = 16) | P |
|---|---|---|---|---|
| Sex | | | | |
| Female | 15 | 9 (50.0%) | 5 (35.3%) | 0.50 |
| Male | 20 | 9 (56.3%) | 11 (64.7%) | |
| Age | | | | |
| 46-60 years | 3 | 1 (5.6%) | 2 (11.8%) | 0.69 |
| >60 years | 32 | 17 (94.4%) | 15 (88.2%) | |
| Multifocality | | | | |
| No | 28 | 15 (83.3%) | 13 (76.5%) | 1.0 |
| Yes | 7 | 3 (16.7%)I | 4 (23.5%) | |
| Grade | | | | |
| Low | 10 | 5 (27.8%) | 5 (29.4%) | 1.0 |
| High | 25 | 13 (72.2%) | 12 (70.6%) | |
| Stage | | | | |
| pTa | 9 | 4 (22.2%) | 5 (29.4%) | 0.40 |
| pT1a | 22 | 13 (72.2%) | 9 (52.9%) | |
| pT1b | 4 | 1 (5.6%) | 3 (17.6%) | |
| p53 | | | | |
| Neg. | 20 | 11 (55.0%) | 9 (45.0%) | 0.73 |
| Pos. | 15 | 7 (46.6%) | 8 (53.3%) | |
| MDM2 | | | | |
| Neg. | 16 | 4 (22.2%) | 12 (70.6%) | 0.007 |
| Pos. | 19 | 14 (77.8%) | 8 (29.4%) | |
| MDM2/p53 | | | | |
| Mdm2+/p53 - | 11 | 8 (44.4%) | 3 (17.6%) | 0.032 |
| Mdm2 -/p53 + | 7 | 1 (5.6%) | 6 (35.3%) | |
| Mdm2 -/p53 – | 9 | 3 (16.7%) | 6 (35.3%) | |
| Mdm2 +/p53 + | 8 | 6 (33.3%) | 2 (11.8%) | |

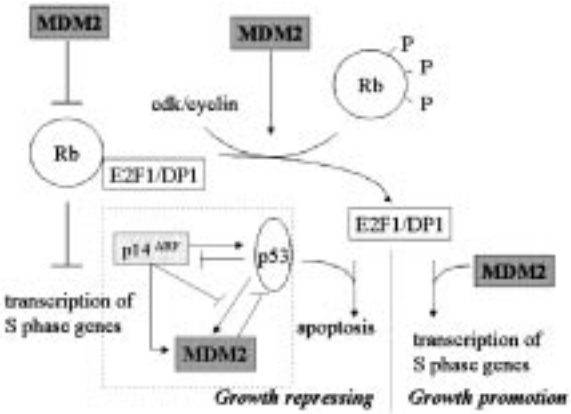


Fig. 2 – Model for MDM2 involvement in the Rb/E2F1 and p53 pathways. MDM2 might prevent E2F1 function during G1- phase transcriptional repression when associated with Rb, influence the Rb/E2F1 interaction itself, or promote the ability of free E2F1 to activate S-phase gene transcription. MDM2 promotes p53 degradation/inactivation, $p14^{ARF}$ inhibit this MDM2 function.

## Discussion

In this study MDM2 overexpression was observed in 51.4% of the cases and an important correlation was demonstrated between MDM2 overexpression assessed by immunohistochemistry and the recurrence rate or recurrence-free survival. The p53 nuclear accumulation, detected in 48.5% of the cases, was not related to prognosis. The concomitant presence between p53 nuclear accumulation and MDM2 overexpression was not significantly related to the recurrence rate. Despite the higher frequency of p53 and MDM2 positive cases in the high grade subgroup, these molecular tests have not significantly distinguished the high grade tumours from low grade superficial ones. The MDM2 positive cases were more frequent in pTa cases (66.7%) contrasting with a decreased frequency of p53 nuclear accumulation in the same cases (33.3%). The current study has a small number of cases studied, which is an important limitation, however the potential importance of the results obtained may deserve discussion according to the actual literature. Lianes and Scmitz-Draguer et al. have found similar results, by reporting an association between MDM2 overexpression and low-grade bladder tumours and a decreased frequency of MDM2 overexpression in the muscle invasive ones (12,14). More recently, Pfister et al. observed that 44% of low-stage tumours presented MDM2 overexpression whereas p53 nuclear accumulation was observed in less than 20% of cases (18). According to these data and the hypothesis of Pfister group, our results, suggests that in a significant proportion of superficial tumours, p53 nuclear accumulation is the result of the altered wild type, which may not permit the action of MDM2-mediated degradation pathway. Jahnson and Korkolopoulou et al. who described MDM2 overexpression in the higher stages obtained different results, but the number of patients with MDM2 overexpression, in both studies, was small. (19-20). Our results indicate that the rate of p53 nuclear accumulation and/or MDM2 overexpression was not significantly different in function of lamina propria (muscularis mucosae) invasion. However Ozdemir et al. showed in pTa-pT1 carcinomas, that nuclear staining of p53, MDM2, or both was highly correlated with the degradation of the basement membrane underlying carcinomas and the expression status of MDM2 should provide better information about the progression of superficial urothelial carcinomas than the status of p53 alone (21). In the present study the cases with MDM2 overexpression were associated with a local recurrence and a significantly lower recurrence-free survival, therefore MDM2 overexpression seems to be a reliable marker to detect potential recurrent superficial papillary bladder tumours. Shiina et al. did not achieve any correlation between MDM2 overexpression and histological grade but a significant better prognosis was detected in MDM2 and p53 negatives tumours (22). Schmitz-Drager et al. showed that MDM2 overexpression alone had no prognostic significance in superficial bladder tumours, however patients with alterations in both genes (MDM2 and TP53) had a very high risk of tumour progression, contrasting with our results (14). In our study the p53 positive and MDM2 overexpressed phenotype was the second one with more number of recurrences and progression, which was not expected in physiologic conditions. Therefore, these recurrences/progression phenotypes (MDM2+/p53+ and MDM2+/p53-) needs further investigation. Different mechanisms are probably the underlying reason of these phenotypes. Mechanisms of stabilisation of p53 occur in response to oncogene signalling, this is thought to result from

Table 3 – Tumour recurrence according to clinical prognostic variables, MDM2+ and p53+ phenotype.

| | Number of cases | OR | 95% CI |
|---|---|---|---|
| Clinicopathological parameters | | | |
| pT1a | 22 | 1.30 | 0.38-4.41 |
| pT1b | 4 | 1.61 | 0.16-16.13 |
| High grade | 25 | 3.42 | 0.68-17.08 |
| Multifocality | 7 | 0.75 | 0.12-4.45 |
| Biological markers | | | |
| p53+ | 15 | 0.27 | 0.05-1.28 |
| MDM2+ | 19 | 7.09 | 1.57-31.96 |

OR- Odds Ratio

increase levels of $p14^{ARF}$, a tumour suppressor protein (9p21- CDKN2a locus) that can form a complex with MDM2 and inhibit its ability to degradate p53 (23--24). Amplification of MDM2 gene may induce oncogenesis by inactivating the wild-type p53 (10,25). Certain human cancers, with excessive MDM2 expression, were achieved through MDM2 gene amplification (25). However, immunohistochemical and western analysis of several human tumours and tumour cell lines showed that some tumours with overexpressed MDM2, did not show amplification of the gene but an increase of transcription or translation (11,26--28). Thus it appears that mechanisms other that not amplification may also result in the overproduction of MDM2. Amplification of MDM2 in bladder cancer is a rare event (12, 29-30). There is also recent evidence, suggesting that MDM2 may play a role in p53-independent pathways by regulating cellular proliferation (Figure2). MDM2 has also recently been shown to interact with the retinoblastoma tumour suppressor protein (pRb) and the E2F-1/DP1 transcription factors (31-32).

E2F-1 transcription factor is a regulator of both cell cycle progression and apoptosis (33). MDM2 overexpression in cellular lines has been shown to inhibit the apoptosis induction by E2F-1 pathways (34). Overexpression of MDM2 results in p53 inhibition and increased Brdu incorporation, PCNA and Ki-67 staining indicative of DNA replication (35). The final result is proliferation induction (28,36). The high proliferation index assessed by Ki-67 has already been established as a prognostic factor in superficial papillary bladder carcinomas (37). Aberrations of p53 and MDM2 are involved in bladder tumorigenesis, MDM2 overexpression but not p53 nuclear accumulation are rarely of high grade, however, a different phenotypes can be observed in superficial tumours (38)

## Conclusion

The MDM2 status is an important marker of cell cycle health. Apparently overexpression of MDM2 is not related with muscular mucosae invasion. It appears to be a prognostic marker in superficial and low grade bladder urothelial carcinomas, thus the establishment of MDM2/p53 phenotype could lead to a better understanding of the outcome of these tumours.

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