Pergamon

*Oral Oncol, Eur J Cancer*, Vol. 31B, No. 5, pp. 328–332, 1995

0964–1955/95 $9.50 + 0.00

0964-1955(95)00034-8

# Infrequent *MDM2* Gene Amplification and Absence of Gross *WAF1* Gene Alterations in Nasopharyngeal Carcinoma

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**We have investigated the possible involvement of *MDM2* and *WAF1* gene alterations in the development of nasopharyngeal carcinoma (NPC). *MDM2* and *WAF1* were analysed in 46 primary NPCs by Southern blot analysis. Forty-five tumours showed a normal *Eco*RI hybridisation pattern and hybridisation intensity with a human *MDM2* cDNA probe. One tumour had more intense normal size *MDM2* hybridising bands. Densitometric scanning revealed a 10–12-fold *MDM2* gene amplification, as compared with human placenta DNA. All 46 tumours showed normal size *WAF1 Eco*RI bands that hybridised with normal intensity. This is the first demonstration of *MDM2* gene amplification in NPC. Nonetheless, our analysis indicates that gross structural alterations of the *MDM2* and *WAF1* genes are infrequent events in the genesis of NPC.**

**Keywords: NPC, *MDM2* amplification, *WAF1***

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## INTRODUCTION

NASOPHARYNGEAL CARCINOMA (NPC) occurs at high frequency in Southern China and Southeast Asia [1], and is the third most common cancer in Southern China after lung and liver cancer [2]. Epstein–Barr virus (EBV) infection and certain genetic and environmental factors have been implicated in the pathogenesis of NPC [3, 4]. In particular, there is a strong association between NPC and EBV [5, 6]. To search for molecular events responsible for the development of NPC, several laboratories have examined the *p53* tumour suppressor gene in this tumour. Immunohistochemical staining showed *p53* overexpression in NPC [7–9]. However, DNA sequence analysis has revealed that more than 90% of NPCs carry wild-type *p53* [10–13].

The *MDM2* gene was originally identified and cloned by virtue of its amplification in a spontaneously transformed, tumorigenic Balb/c fibroblast cell line [14]. The product of the *MDM2* gene, p90, forms a tight complex with both wild-type and mutant *p53* [15, 16], and inhibits wild-type *p53*-mediated transactivation by masking the *N*-terminal acidic transactivating domain of *p53* [17]. *MDM2* gene amplification has been found in most types of human sarcomas [18–20], and in a subset of human glioblastomas and anaplastic astrocytomas [21]. In addition, oestrogen receptor-positive breast carcinoma cell lines were found to express elevated levels of *MDM2* mRNA [22]. Therefore, amplification and/or overexpression of *MDM2* may represent an alternative mechanism for inactivation of wild-type *p53* function.

*WAF1* (also designated CIP1) was recently identified as a downstream effector of *p53* [23, 24]. The *WAF1* promoter contains two *p53* consensus binding sites and is transactivated by wild-type *p53*. The *WAF1* gene product, p21, binds and blocks the activity of cyclin–cdk complexes [25], and thereby induces G1 arrest or apoptosis. It seems possible that inactivation of *WAF1* by gross rearrangement or point mutation could at least partially abolish the normal *p53*-mediated growth control pathway.

Given the low frequency of *p53* mutations in NPC, we asked whether functional inactivation of *p53* by *MDM2* amplification or *WAF1* deletion or rearrangement occurred frequently in this tumour. We found *MDM2* gene amplification in one out of 46 primary NPCs examined, whereas all tumours had normal size *WAF1* hybridising bands. These results show that inactivation of *p53* function by *MDM2* amplification or *WAF1* deletion or rearrangement does not play a major role in the development of NPC.

## MATERIALS AND METHODS

### *Tumours*

Forty-six NPC biopsies were obtained from different clinically diagnosed patients at the Shanghai Cancer Hospital, the Ear–Nose–Throat Hospital, and the Guanzhou provincial hospital, prior to treatment. Biopsy specimens were snap-frozen within 1 h of surgical removal and classification. Diagnosis was based on histopathological examination, per-

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Received 26 Jan. 1995; provisionally accepted 20 Feb. 1995; revised manuscript received 18 Apr. 1995.

formed by the pathologist at each hospital according to the WHO classification [26]. All tumours were undifferentiated carcinomas with no evidence of squamous differentiation, and were EBV positive as shown by hybridisation with an EBV–LMP1 probe or by Western blotting with the polyclonal anti-EBNA-1 antibody PG and the monoclonal anti-LMP1 antibody S12 [27].

*Southern blot analysis*

High molecular weight DNA was prepared from frozen tissue samples as described [28]. Five micrograms of DNA from each biopsy were digested overnight with 25 units of *Eco*RI, *Xba*I, or *Hind*III restriction endonucleases in the appropriate buffer. After electrophoretic separation in a 0.8% agarose gel run in TAE buffer (0.04 M Tris–acetate, 1 mM EDTA), the DNA was transferred to nylon filters (Hybond-N, Amersham). The filters were hybridised with a 32-P-labelled 0.9 kb *Xho*I fragment of human *MDM2* cDNA or a 2.1 kb *Bam*HI–*Hind*III fragment containing human *WAF1* cDNA. *MDM2* copy number was estimated using densitometry and normalised to a single copy control gene (human α-actin). The human osteosarcoma cell line SJSA-1 (originally termed OsA-CL) which carries a 5–50-fold amplification of *MDM2* [18] served as a positive control.

## RESULTS

Forty-six primary NPC biopsies were tested for *MDM2* gene amplification by Southern blot analysis. As shown in Fig. 1a and b, the *MDM2* probe hybridised with normal size *Eco*RI fragments in all tumours. The intensity of the *MDM2*-hybridising bands was comparable to that of the *MDM2* bands in human placenta DNA in all tumours except one. In this tumour, NPC 9 (Fig. 1a, lane 14), the *MDM2* *Eco*RI fragments hybridised with an intensity similar to the *MDM2*

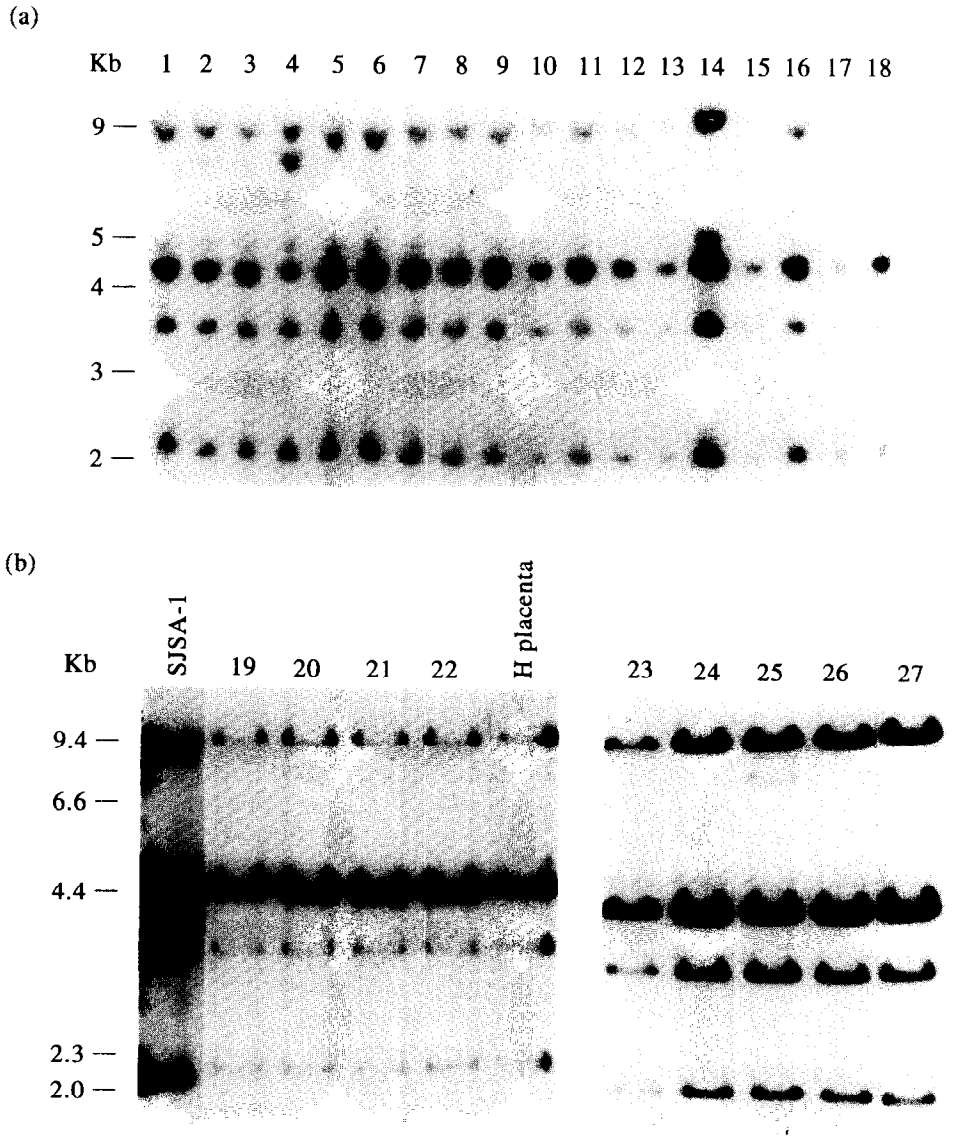


**Fig. 1. Southern blot analysis of the *MDM2* gene in NPC. *Eco*RI-digested DNA from each tumour specimen was hybridised with a human *MDM2* cDNA fragment probe as described in Materials and Methods. SJSA-1, a human osteosarcoma cell line previously shown to have 5–50-fold amplification of the *MDM2* gene, was used as a positive control. DNA from normal human placenta was used as a negative control. DNA fragment sizes are shown in kb. (a) and (b) show the results for 27 out of the 46 NPCs examined. One tumour, designated NPC9, shows a 10–12-fold *MDM2* gene amplification.**

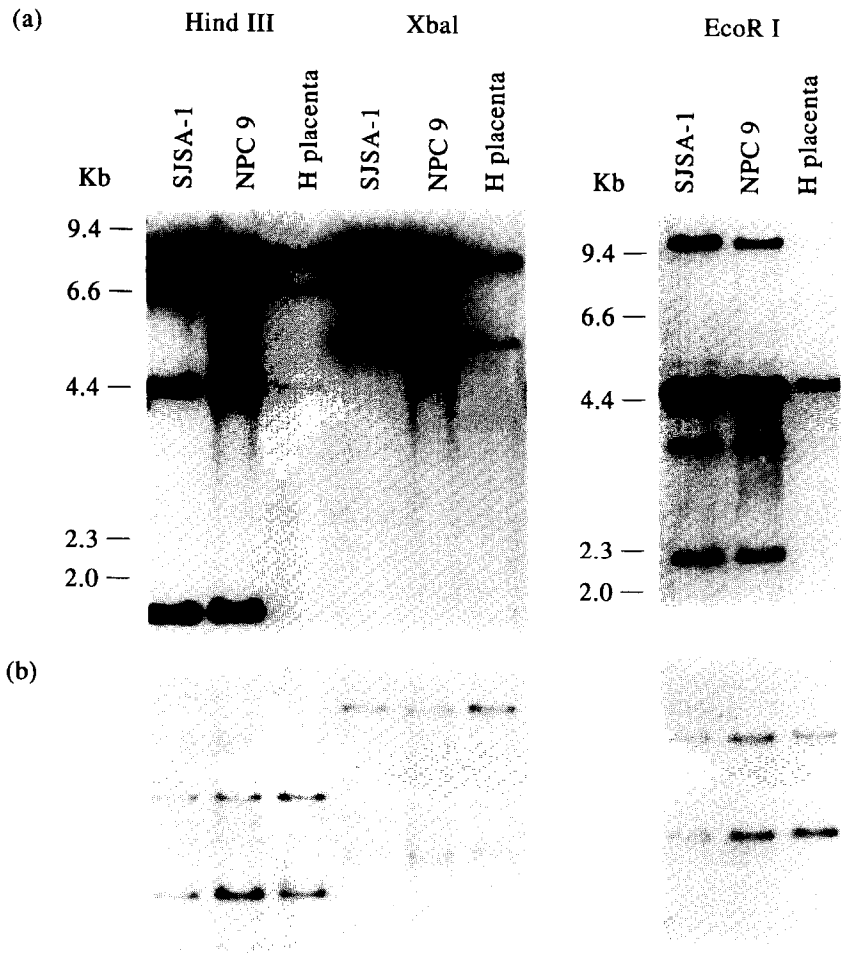


**Fig. 2. Southern blot analysis of the *MDM2* gene in NPC9. Samples were digested with *Hind*III, *Xba*I or *Eco*RI. DNA fragment sizes are shown in kb. (a) Hybridisation with the *MDM2* probe. (b) Hybridisation of the same filters with an α-actin control probe.**

fragments in SJSA-1, the human osteosarcoma cell line used as a positive control. Digestion with other restriction enzymes (*Xba*I, *Hind*III) confirmed *MDM2* gene amplification without rearrangement in NPC 9 (Fig. 2a). According to densitometry scanning, the *MDM2* gene is amplified 10–12-fold in NPC 9, relative to human placenta.

In order to confirm that the amplification of *MDM2* in NPC 9 was not due to unequal amounts of loaded DNA, the filters shown in Fig. 2a were rehybridised with a single copy control probe, human α-actin. This probe hybridised with equal intensity to the DNA from NPC9, SJSA-1 and human placenta (Fig. 2b), demonstrating that approximately equal amounts of NPC 9, SJSA-1 and human placenta DNA had been loaded.

The same filters were also hybridised with a human *WAF1* probe. This probe detected *Eco*RI fragments of approximately 5.5. and 12 kb in human placenta DNA, as well as in DNA from all 46 tumours, and hybridised with equal intensity in all DNA samples (Fig. 3; data shown for six NPCs, including NPC9). Thus, none of the tumours had major rearrangement or amplification of *WAF1*.

## DISCUSSION

Previous work in several laboratories has shown that inactivation of *p53* by point mutation does not play a significant role in the development of NPC. These studies demonstrated *p53* mutation in 10% or less of the tumours [10–13]. Similarly, we have sequenced exons 4–8 of *p53* in 11 NPCs and found only one tumour with mutant *p53* (K.P. Magnusson & E. Kashuba, personal communication). Inactivation of wild-type p53 function may also occur at the protein level, through complexing with the cellular MDM2 protein [17]. Amplification of *MDM2* has been found in human sarcomas, glioblastomas and anaplastic astrocytomas [18–21]. Therefore, it appeared important to examine whether *MDM2* amplification and/or overexpression had contributed to inactivation of wild-type *p53* in NPC. One out of 46 primary NPCs showed 10–12-fold *MDM2* gene amplification, comparable to the degree of *MDM2* amplification in the human osteosarcoma line SJSA-1. This is the first example of *MDM2* amplification in NPC described so far. This NPC carries wild-type *p53* according to a DNA sequence analysis of exons 4–8 (K.P. Magnusson & E. Kashuba, personal communication).

Nevertheless, our results demonstrate that *MDM2* gene amplification is not a common mechanism for inactivation of the *p53* pathway in NPC, since the great majority of tumours carried single copy *MDM2*. Furthermore, we did not find any evidence for inactivation of *WAF1* by deletion or rearrangement, although it remains possible that *WAF1* has been inactivated by smaller deletions or point mutations undetectable by the Southern blot analysis applied here. These

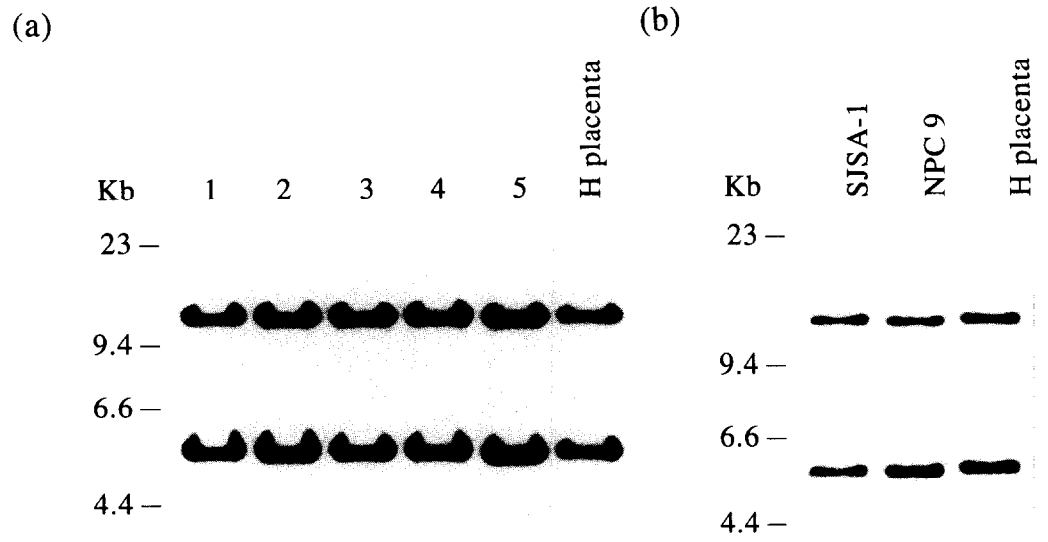


**Fig. 3. Southern blot analysis of the *WAF1* gene in NPC. *Eco*RI-digested DNA samples were hybridised with a human *WAF1* probe as described. DNA fragment sizes are shown in kb. (a) Results for five of the 46 NPC biopsies tested. (b) Results for SJSA-1, NPC9 and human placenta DNA.**

observations and the fact that *p53* is infrequently mutated in NPC suggest that loss of *p53* function is not important for the development of NPC. Alternatively, *p53* inactivation may have occurred by other mechanisms. Several viral oncoproteins can bind *p53* and block its transactivating activity [29, 30]. The close association between EBV and NPC [5, 6] raises the possibility that a protein or proteins encoded by EBV contribute to nasopharyngeal carcinogenesis by inhibiting the normal function of *p53*. We have previously shown that the EBV-encoded EBNA-5 protein can complex with *p53 in vitro* [31]. However, EBNA-5 is unlikely to contribute to NPC development since it is not expressed in NPC as a rule [27, 32]. Another possibility is that the EBV-encoded LMP1 protein that is expressed in a majority of NPCs [27] can in some way interfere with *p53* function.


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**Acknowledgements**—The authors thank Dr Bert Vogelstein, Johns Hopkins University, for providing human *MDM2* and *WAF1* cDNA, and Dr Tom Look, St. Jude Children's Hospital, for the human osteosarcoma cell line SJSA-1. This work was supported by the Swedish Cancer Society (Cancerfonden; project no. 3336-B93-02XBB) and by Concern Foundation/Cancer Research Institute.