

The nm23-H1 gene as a predictor of sensitivity to chemotherapeutic agents in oesophageal squamous cell carcinoma

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Summary Recently, nm23-H1, an anti-metastasis gene, has been reported to correlate with sensitivity to chemotherapeutic agents including cisplatin in human breast and ovarian carcinoma cells. The aim of this study was to evaluate a role for nm23-H1 in responsiveness to cisplatin-based chemotherapy in patients with oesophageal squamous cell carcinoma (OSCC). The expression of nm23-H1 protein was examined immunohistochemically in 32 eligible patients with OSCC who underwent adjuvant chemotherapy with cisplatin, etoposide, and 5-fluorouracil after tumour resection. Fifteen (46.9%) of 32 patients were positive for nm23-H1 staining and 17 (53.1%) were negative. Both disease-free survival and overall survival rates of nm23-H1-negative patients were significantly shorter than in nm23-H1-positive patients ($P < 0.01$ for both). There was no significant difference in clinicopathologic characteristics between nm23-H1-positive and nm23-H1-negative groups. Multivariate analysis also showed that nm23-H1 expression was the most significant factor for overall survival of OSCC patients included in this study ($P = 0.0007$). To further study the role of nm23-H1, a human OSCC cell line (YES-2) was transfected with a plasmid containing a fragment of the nm23-H1 cDNA in an antisense orientation. Reduced expression of nm23-H1 protein in the antisense-transfected (AS) clones was found by Western blot analysis as compared to wild-type YES-2 and YES-2/Neo (clone transfected with the neomycin resistance gene alone). MTT (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide) assay showed that reduced expression of the nm23-H1 protein in AS clones was consistent with the degree of increased resistance to cisplatin but not etoposide or 5-fluorouracil. These data support the conclusion that reduced expression of nm23-H1 may be associated with resistance to cisplatin, suggesting the value of nm23-H1 expression as a prognostic marker for OSCC patients who are to undergo cisplatin-based chemotherapy. © 1999 Cancer Research Campaign

Keywords: nm23; cisplatin; chemotherapy; prognosis; oesophageal squamous cell carcinoma

About half of all patients with oesophageal squamous cell carcinoma (OSCC) have locoregional disease at diagnosis; however, 40–50% of them die of metastatic relapse within the first 2 years after tumour resection (Roder et al, 1994). Thus, the prognosis of patients with locally advanced OSCC remains poor despite significant progress in surgical treatment. Several regimens of cisplatin-based chemotherapy and/or chemoradiotherapy have been applied to treatment of patients with OSCC (Kok et al, 1996; Stahl et al, 1996; Bosset et al, 1997); however, these clinical trials have not shown significant improvement in overall survival so far. These results mean that it may be necessary to distinguish responders from non-responders in cisplatin-based chemotherapy in order to determine a more effective therapy and to improve the poor prognosis of this disease.

The nm23 gene was identified originally as an anti-metastatic influence whose expression was correlated inversely with tumour metastatic potential in murine melanoma cell lines (Steege et al, 1988). Subsequently, several studies have demonstrated the favourable clinical outcome of overexpression of nm23-H1 in human malignant tumours (Florenes et al, 1992; Tokunaga et al, 1993; Iizuka et al, 1995), although conflicting data have been

reported (Higashiyama et al, 1992; Easty et al, 1996; Lindmark et al, 1996). More interestingly, nm23-H1 has been shown to relate to sensitivity to cisplatin in several malignant tumours (Ferguson et al, 1996; Scambia et al, 1996; Freije et al, 1997). Our preliminary study also showed that reduced expression of nm23-H1 protein was associated with poor prognosis of patients with OSCC. However, we observed that there was a lack of association between the expression and prognosis of patients with OSCC who did not undergo cisplatin-based chemotherapy (data not shown). Therefore, in the present study, we focused on a role for nm23-H1 in responsiveness to cisplatin-based chemotherapy in OSCC patients, and we examined nm23-H1 expression in 32 primary tumours of OSCC patients who underwent the same protocol of cisplatin-based chemotherapy following tumour resection. Furthermore, we evaluated the direct correlation between tumour nm23-H1 expression and resistance and/or sensitivity to chemotherapeutic agents including cisplatin using an antisense-transfection assay.

MATERIALS AND METHODS

Patients

Between April 1989 and July 1996, we had performed cisplatin-based chemotherapy following resection in 35 OSCC patients with stage II–IV defined by the TNM classification of the International Union Against Cancer (1997). Of the 35 patients two died of subarachnoid haemorrhage and myocardial infarction during

Received 17 September 1998

Revised 1 February 1999

Accepted 21 April 1999

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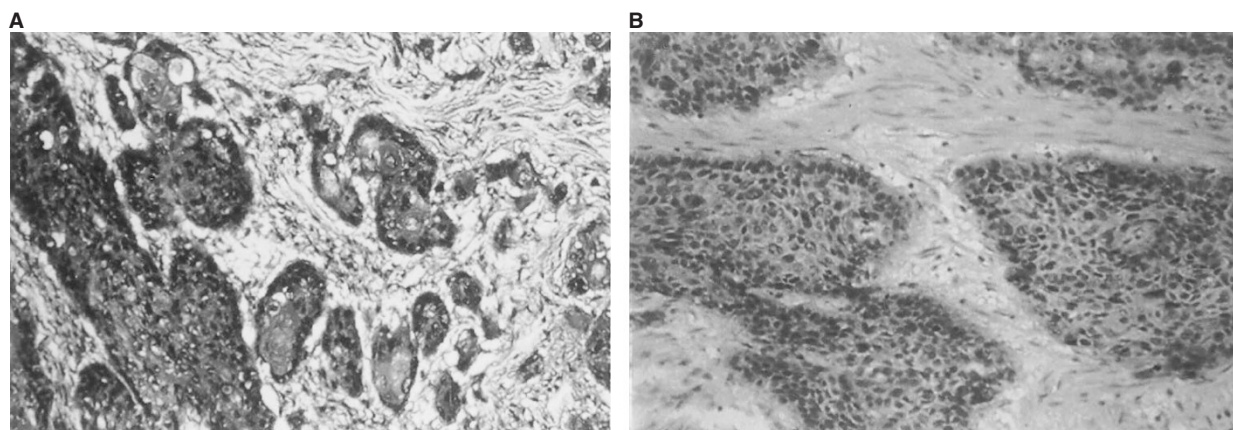


Figure 1 Immunohistochemical staining for nm23-H1 protein in oesophageal squamous cell carcinoma. (A) Representative nm23-H1-positive case which demonstrates intense nm23-H1 immunoreactivity in the cytoplasm of tumour cells (original magnification $\times 100$). (B) Representative nm23-H1-negative case in which expression in tumour cells was similar to that of nontumorous tissue (original magnification $\times 200$)

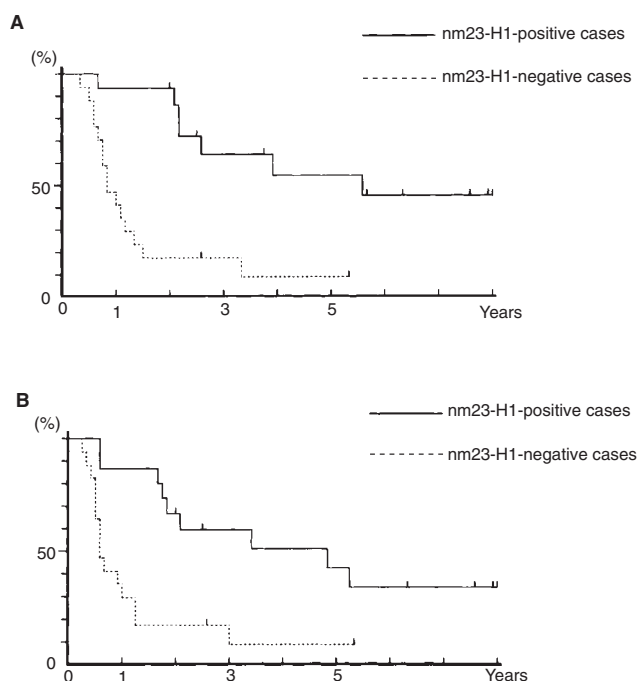


Figure 2 Overall survival (A) and disease-free survival (B) curves of patients with OSCC according to nm23-H1 status

follow-up periods respectively. One patient was excluded because of loss to follow-up. Therefore, the present study was undertaken in 32 patients retrospectively selected. One course of cisplatin-based chemotherapy consisted of etoposide 120 mg m^{-2} per day and 5-fluorouracil (5-FU) 500 mg m^{-2} per day by intravenous (i.v.) continuous infusion on days 3–5, and cisplatin 50 mg m^{-2} per day by i.v. bolus infusion on days 1 and 8. Seven (21.9%) of 32 patients underwent only 1 course of this regimen because of side-effects, while 25 patients (78.1%) underwent two course of this regimen following resection. All patients were subjected to computerized tomography and magnetic resonance imaging every 3 months. These imaging techniques revealed metastatic relapse in

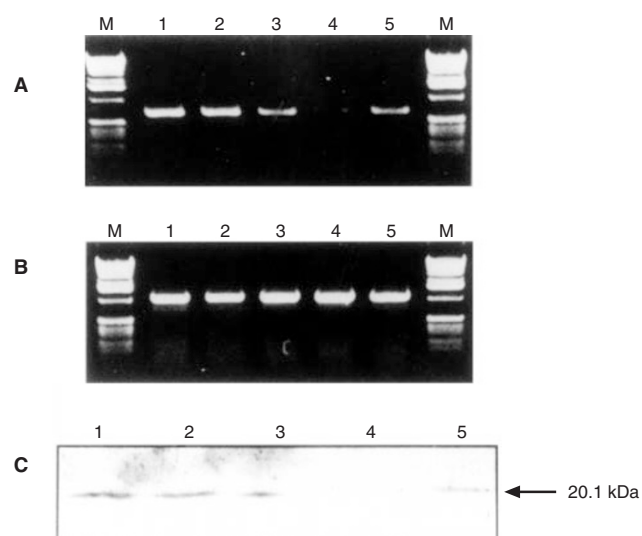


Figure 3 Down-regulation of both intrinsic mRNA and protein level of nm23-H1 by antisense nm23-H1 cDNA transfection. (A) Intrinsic nm23-H1 mRNA expression detected by RT-PCR with primers which yield a 685-bp product and can exclude exogenously transfected antisense nm23-H1 cDNA. (B) Expression of β -actin gene (internal control) amplified simultaneously by RT-PCR. (C) Expression of nm23-H1 protein by Western blot analysis with mAb H1-229. Lanes 1–5 are YES-2, YES-2/Neo, YES-2/AS-5, YES-2/AS-12 and YES-2/AS-29 respectively. Relative expression of nm23-H1 protein in YES-2/Neo, YES-2/AS-5, YES-2/AS-12 and YES-2/AS-29 was 0.92, 0.56, 0.04 and 0.41 respectively, when compared to that of YES-2. Lane M is a molecular weight marker, 1 kDa DNA ladder (Gibco-BRL, Rockville, MD, USA)

24 (75%) of the 32 patients, and 23 of the 24 patients died of metastatic relapse within the median follow-up period of 65 months (range 21–105 months).

Immunohistochemical analysis for NM23-H1 protein

The expression of nm23-H1 protein was examined immunohistochemically in 32 primary tumours of 32 eligible patients. Following resection, tumour samples were fixed in 10% formaldehyde solution and embedded in paraffin. Four-micrometre-thick sections were

Table 1 nm23-H1 expression and clinicopathologic characteristics

Factors	nm23-H1(+)	nm23-H1(-)	P-value
Years			NS
<60	7	11	
≥60	8	6	
Sex			NS
Male	13	15	
Female	2	2	
Location			NS
Upper thoracic	1	3	
Mid-thoracic	11	8	
Lower thoracic	3	6	
pT			NS
pT1	0	2	
pT2	3	3	
pT3	7	7	
pT4	5	5	
pN			NS
pN0	2	1	
pN1	13	16	
pM			NS
pM0	12	12	
pM1	3	5	
pTNM stage			NS
II	4	5	
III	8	7	
IV	3	5	
Differentiation			NS
Well	2	2	
Moderate	9	10	
Poor	4	5	
Venous invasion			NS
(+)	9	9	
(-)	6	8	
Lymphatic invasion			NS
(+)	14	13	
(-)	1	4	
Resectability			NS
R0	6	5	
R1	4	7	
R2	5	5	
EFP therapy			NS
1 course	3	4	
2 course	12	13	

NS, not significant; EFP, etoposide, 5-fluorouracil and cisplatin.

deparaffinized in xylene and progressively rehydrated in decreasing concentrations of alcohol. Immunohistochemical staining was performed by the avidin-biotin affinity method with the use of OmniTags (Lipshaw, Pittsburgh, PA, USA). Briefly, the sections were immersed in protein blocking agent for 5 min to reduce non-specific staining and incubated at room temperature for 120 min with 1 µg ml⁻¹ anti-human nm23-H1 monoclonal antibody (H1-229, Seikagaku, Tokyo, Japan) (Tokunaga et al, 1993; Iizuka et al, 1995). These sections were washed in chilled PBS three times and were reacted with biotinylated polyvalent antibody at room temperature for 30 min. After being washed in PBS, the sections were reacted with streptavidin-alkaline phosphatase reagent at room temperature for 30 min. Finally, the sections were treated with fast red chromogen for 10 min. Normal mouse IgG was used as a negative control instead of the primary antibodies. The sections were counterstained lightly with haematoxylin. We judged that nm23-H1 protein was positive when more than 20% of the cancer cells were more strongly stained than stromal cells (Figure 1).

Antisense vector for nm23-H1 cDNA

Nm23-H1 cDNA was isolated by reverse transcription polymerase chain reaction (RT-PCR) using total RNA purified from human peripheral blood mononuclear cells. The primers for nm23-H1 were as follows: sense primer, 5'-GCGTCTAGAGGAACCATGGC-CAACTGTGAGCGT-3' and antisense primer, 5'-GTCGCGGCC-GCTCTGCCCTCCTGTCATTCATAGAT-3'. The PCR fragment was cloned into a *NotI/XbaI* site of the pcDNA II plasmid (Invitrogen, San Diego, CA, USA), and the nucleotide sequence was confirmed by automatic DNA sequencer DSO-1000 (Shimazu, Kyoto, Japan). The same fragment then was recloned into a *NotI/XbaI* site of the pRc/CMV eukaryotic expression vector (Invitrogen, San Diego, CA, USA).

Tumour cells and transfection

YES-2 cells, a human OSCC cell line (Oka et al, 1996; Yamamoto et al, 1997), were maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal serum, 100 U ml⁻¹ penicillin G, and 100 µg ml⁻¹ streptomycin. Subconfluent cultures in 100 mm Petri dishes were transfected with 5 µg of antisense nm23-H1 expression plasmid or vector alone using the lipofectAMINE reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. G418 (600 µg ml⁻¹) (Life Technologies, Rockville, MD, USA) was added to the cells 48 h later. G418-resistant clones were isolated and expanded in culture medium containing 200 µg ml⁻¹ of G418.

Effects of antisense transfection on intrinsic nm23-H1 mRNA

The RT-PCR method was performed as previously described (Iizuka et al, 1995). Briefly, cDNA was synthesized from 0.5 µg of total RNA using random hexadeoxynucleotide primers (Takara, Otsu, Japan) and was amplified using AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, San Diego, CA, USA). The primers used to detect only intrinsic nm23-H1 mRNA were 5'-GCAGCCGGAGTTCAAACC-TAA-3' (sense) and 5'-GCTGGGAGGAAGCATTTTAATCA-3' (antisense), which yielded a 685-bp product and can exclude exogenously transfected antisense nm23-H1 cDNA. The primers for the β-actin gene were 5'-ATGGATGATGATATCGCCGCGCT-3' (sense) and 5'-CGGACTCGTCATACTCCTGCTTG-3' (antisense), which yielded a 1224-bp product. PCR reaction was performed for 27 cycles according to the following parameters: denaturation at 94°C for 1 min, annealing at 55°C for 45 s, extension at 72°C for 2 min. The PCR products were separated electrophoretically on a 1% agarose gel and stained with ethidium bromide.

Western blot analysis

Western blot analysis was also performed according to previously described method (Iizuka et al, 1995). Briefly, protein samples (50 µg) were subjected to 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto nitrocellulose filters. The antibody used was monoclonal antibody (H1-229) which is specifically directed against NM23-H1 protein (Tokunaga et al, 1993). For detection of the immunocomplex, the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Tokyo, Japan) was used. Finally, the membrane was exposed to XAR-5 film (Eastman-Kodak, Rochester, NY, USA) for 30 s at room temperature.

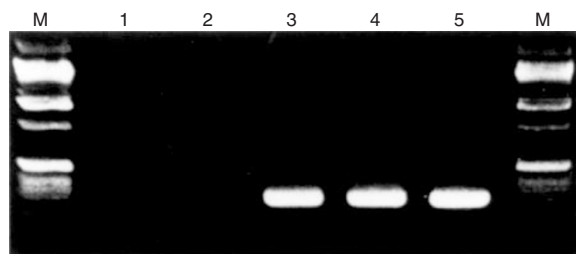


Figure 4 PCR analysis of genomic DNA extracted from AS transfectants, YES-2/Neo, and YES-2 parental cells for the presence of a plasmid expressing antisense nm23-H1 cDNA fragment. Lanes 1–5 are YES-2, YES-2/Neo, YES-2/AS-5, YES-2/AS-12 and YES-2/AS-29 respectively. Lane M is a molecular weight marker, 1 kB DNA ladder (Gibco-BRL, Rockville, MD, USA)

Analysis of integration of antisense nm23-H1 cDNA

We used a PCR method to confirm the integration of antisense nm23-H1 cDNA into YES-2 cells by previously reported methods (de Geovani et al, 1996). PCR was performed on genomic DNA extracted by DNazol (Life Technologies, Rockville, MD, USA), and 0.5 µg was used for each PCR reaction in 100 µl final volume. The following primers were used to detect antisense nm23-H1 cDNA fragment of the pRc/CMV eukaryotic expression vector: sense primer, 5'-GAGATTATCAAGCGTTTTGAGCA-3' (position 151–173, exon 2) and antisense primer, 5'-GTATAATGTTCTGCCAACTTGT-3' (position 436–414, exon 4–5), which yielded a 286-bp product. PCR reaction was performed for 30 cycles according to the following parameters: denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 90 s.

Assessment for in vitro cytotoxicity induced by chemotherapeutic agents

Cisplatin and etoposide were purchased from Nippon Kayaku (Tokyo, Japan), whereas 5-FU was obtained from Kyowa Hakko (Tokyo, Japan). MTT (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide; Dojindo, Tokyo, Japan) assay was performed to evaluate the in vitro cytotoxicity induced by cisplatin, etoposide and 5-FU in YES-2, YES-2/Neo, and AS clones. The cells were plated at 5×10^3 per 100 µl in 96-well plates in Dulbecco's modified Eagle's medium with 5% fetal calf serum, and allowed to attach overnight. Three wells were treated with each drug concentration of 0.01 µg ml⁻¹ to 20 µg ml⁻¹ of cisplatin or etoposide or 0.01 µg ml⁻¹ to 50 µg ml⁻¹ of 5-FU. After 48 h, cell viability was calculated as the percentage of control cultures which were not exposed to drugs. Thus, the experiments were performed in triplicate and the results were evaluated using the means of three experiments.

Statistical analysis

The χ^2 test with Yates' correction or Fisher's exact test was used to elucidate the correlation between nm23-H1 expression and clinicopathological characteristics. All survival and disease-free survival data were analysed by the Kaplan–Meier method using Cox–Mantel comparisons for statistical significance (Kaplan and Meier, 1958; Cox, 1972). Multivariate analysis by the Cox proportional hazards regression model was performed to evaluate the factors responsible for overall survival of OSCC patients who

Table 2 Factors linked to overall survival

Factors coefficient	Regression error	Standard	t-value	P-value
Nm23-H1 staining	2.1023	0.5549	3.789	0.0007
Sex	1.9963	0.6733	2.920	0.0068
Tumour location	0.3590	0.3091	1.162	0.2552

Table 3 In vitro inhibitory effect of cisplatin on control- and antisense nm23-H1 transfected tumour cell lines

Cell line	nm23-H1 protein level	ID ₅₀ (µg ml ⁻¹)
YES-2	1.00	1.3
YES-2/Neo	0.92	1.1
YES-2/AS-5	0.56	2.1
YES-2/AS-12	0.04	4.1
YES-2/AS-29	0.41	2.0

ID₅₀, the cisplatin dose at which the cell number is 50% of control (untreated) cultures as determined by MTT assay.

underwent cisplatin-based chemotherapy after tumour resection. Pearson's correlation test was used to analyse the relationship between nm23-H1 protein level and ID₅₀ of cisplatin in YES-2 and the antisense transfectants.

RESULTS

Immunohistochemical study

Of 32 patients included in this study, 15 (46.9%) were positive for nm23-H1 staining and 17 (53.1%) were negative. In 15 (88.2%) of 17 nm23-H1-negative cases, the intensity of nm23-H1 immunostaining of the primary tumour was weaker than corresponding normal oesophageal epithelium (data not shown). The disease-free survival and overall survival curves for patients with positive or negative nm23-H1 staining are shown in Figure 2. Overall 1-, 3- and 5-year survival rates of nm23-H1-negative patients were 41.2%, 17.6% and 8.8% respectively. By contrast, those of nm23-H1-positive patients were 93.3%, 63.8% and 54.7% respectively. Disease-free 1-, 3- and 5-year survival rates of nm23-H1-negative patients were 29.4%, 8.8% and 8.8% respectively, while those of nm23-H1-positive patients were 86.7%, 59.3% and 42.3% respectively. Thus, nm23-H1-negative patients had both significantly shorter disease-free survival and overall survival than did nm23-H1-positive patients (Figure 2, $P < 0.01$ for both). Multivariate analysis also showed that, of ten factors examined, nm23-H1 expression was the most significant factor for overall survival of OSCC patients who underwent cisplatin-based chemotherapy after tumour resection ($P = 0.0007$, Table 1). Of the ten OSCC patients who had poor resectability (R2 resection), two (40%) of five patients with positive nm23-H1 staining are alive in condition of relapse-free after this chemotherapy, on the contrary, all of the five patients with negative nm23-H1 staining died of metastatic relapse. As shown in Table 2, there was no significant difference in the other clinicopathological characteristics between nm23-H1-positive and nm23-H1-negative groups. No association was observed between nm23-H1 expression and local immune response such as lymphocyte infiltration (data not shown).

In vitro cytotoxic effect of chemotherapeutic agents on control- and antisense nm23-H1-transfected cells

Transfection of antisense nm23-H1 cDNA was performed to evaluate the relation of nm23-H1 to cytotoxicity induced by chemotherapeutic agents in OSCC cells. Of 30 G418-resistant clones isolated in culture medium containing $600 \mu\text{g ml}^{-1}$ of G418, our RT-PCR method selected these clones, YES-2/AS-5, YES-2/AS-12 and YES-2/AS-29, which showed reduced expression of intrinsic nm23-H1 mRNA (Figure 3). Subsequently, the nm23-H1 protein level in these clones was analysed by Western blot analysis (Figure 3). Relative expression of nm23-H1 protein in YES-2/AS-5, YES-2/AS-12, YES-2/AS-29 and YES-2/Neo calculated by densitometer was 0.56, 0.04, 0.41 and 0.92 respectively, when compared to that of YES-2 cells (parental cell). Furthermore, the integration of antisense nm23-H1 cDNA into YES-2/AS-5, YES-2/AS-12 and YES-2/AS-29 was confirmed by PCR analysis with genomic DNA extracted from these cells (Figure 4). Namely, a spliced 286-bp product was amplified from genomic DNA of the AS clones as well as from a plasmid DNA, but not from YES-2 and YES-2/Neo cells.

We used MTT assay in order to evaluate in vitro cytotoxicity induced by cisplatin, etoposide and 5-FU. There was no difference in growth rates between the AS clones and YES-2 or YES-2/Neo (data not shown). Increased resistance of the AS clones to cisplatin was observed when compared to YES-2 or YES-2/Neo (Figure 5). The ID_{50} of YES-2/AS-5, YES-2/AS-12, YES-2/AS-29 and YES-2/Neo for cisplatin treatment was 2.1, 4.1, 2.0 and $1.1 \mu\text{g ml}^{-1}$ respectively. A reduction of twofold in the expression level of nm23-H1 by YES-2/AS-5 and YES-2/AS-29 resulted in twofold increased resistance to cisplatin as compared to YES-2/Neo. Furthermore, a reduction of 23-fold in the expression level of nm23-H1 by YES-2/AS-12

resulted in fourfold increased resistance to cisplatin as compared to YES-2/Neo (Table 3). Thus, nm23-H1 protein level in the five cells including the parental cell was associated inversely with ID_{50} of CDDP ($r = -0.935$, $P < 0.02$). By contrast, there was no difference in resistance to etoposide or 5-FU between AS clones and YES-2/Neo, although YES-2/AS-12, with markedly reduced expression of nm23-H1 protein, showed increased resistance to 5-FU (Figure 5).

DISCUSSION

Cisplatin is one of the key drugs for treatment of patients with OSCC (Kok et al, 1996). However, cisplatin-based chemotherapy dose not sufficiently improve their overall survival, even when combined with additional irradiation (Stahl et al, 1996; Bosset et al, 1997). The results of these clinical trials suggest that it might be necessary to select the most effective therapy for each case in order to improve survival of patients with OSCC, especially advanced OSCC. Recent studies have elucidated several molecules, such as p53, GST- π and metallothionein, responsible for sensitivity to cisplatin (Timmer-Boscha et al, 1993; Rusch et al, 1995; Hishikawa et al, 1997). More recently, nm23-H1 has been reported to correlate directly with sensitivity to cisplatin as well as other alkylating agents by transfection assay (Ferguson et al, 1996) and the expression of nm23-H1 protein has been shown to correlate inversely with prognosis of patients with ovarian carcinoma after cisplatin-based chemotherapy (Scambia et al, 1996).

With regard to OSCC, work by Patel et al (1997) has shown that reduced expression of nm23 protein was a factor associated with poor prognosis of OSCC patients after resection; however, they did not refer to the relation of nm23 expression to drug sensitivity.

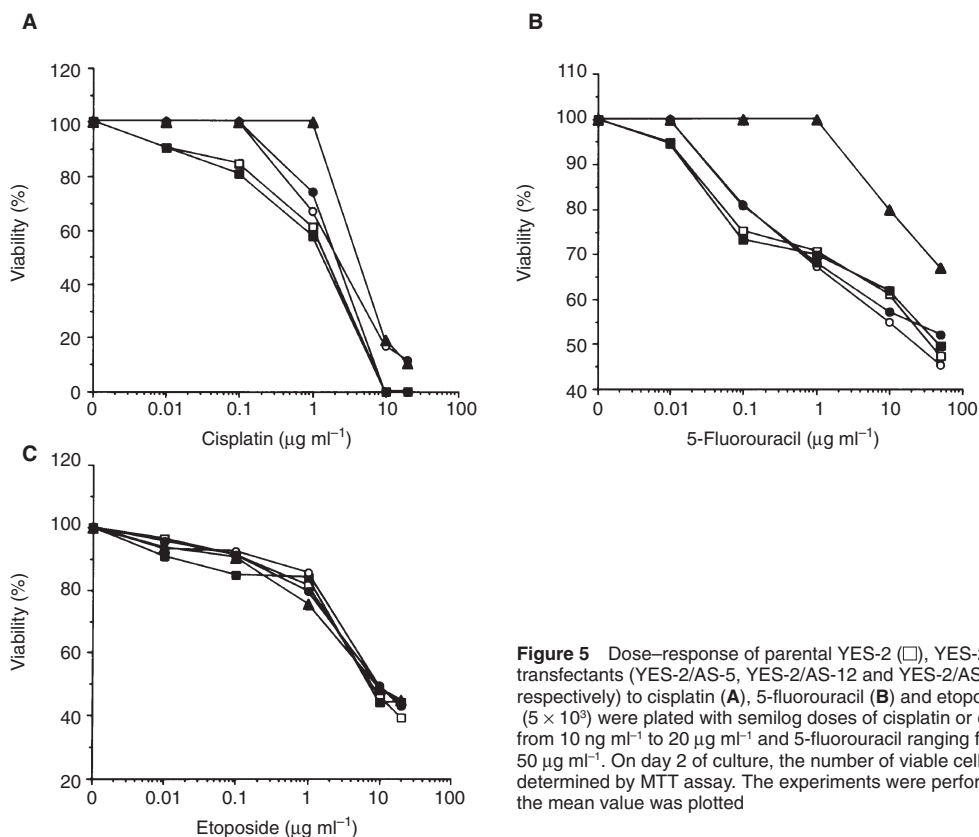


Figure 5 Dose-response of parental YES-2 (□), YES-2/Neo (■), and AS transfectants (YES-2/AS-5, YES-2/AS-12 and YES-2/AS-29, ○, ▲ and ● respectively) to cisplatin (A), 5-fluorouracil (B) and etoposide (C). Cells (5×10^3) were plated with semilog doses of cisplatin or etoposide ranging from 10 ng ml^{-1} to $20 \mu\text{g ml}^{-1}$ and 5-fluorouracil ranging from 10 ng ml^{-1} to $50 \mu\text{g ml}^{-1}$. On day 2 of culture, the number of viable cells per well was determined by MTT assay. The experiments were performed in triplicate and the mean value was plotted

Our immunohistochemical study showed that expression of nm23-H1 protein was correlated inversely with disease-free survival and overall survival rates of OSCC patients who underwent cisplatin-based chemotherapy following resection. Multivariate analysis showed that nm23-H1 status was most responsible for overall survival of OSCC patients after cisplatin-based chemotherapy. Furthermore, we confirmed that, of the ten OSCC patients who had poor resectability (R2 resection), two (40%) of five patients with positive nm23-H1 staining were alive and relapse free after this chemotherapy, on the contrary, all of the five patients with negative nm23-H1 staining died of metastatic relapse. Thus, nm23-H1 is likely to relate to responsiveness to cisplatin-based chemotherapy in patients with OSCC. Stahl et al (1996) have reported that overall 3-year survival rate was 33% in patients with T2–4 oesophageal carcinoma who underwent preoperative chemoradiotherapy and surgery. Likewise, we found that overall 3-year survival rate of 32 OSCC patients included in this study was 39.6%. However, that of 15 OSCC patients with positive nm23-H1 staining was 63.8% after this chemotherapy. These results suggest that nm23-H1 status may be a useful marker for cisplatin-based chemotherapy in patients with OSCC.

MTT assay showed enhanced resistance to cisplatin, but not etoposide or 5-FU, in parallel to the degree of reduction of nm23-H1 protein level in the OSCC cells examined. This result was most consistent with the data reported by Freije et al (1997), except for 5-FU. The role of nm23-H1 in cisplatin-induced cytotoxicity remains to be clarified. Ferguson et al (1996) have demonstrated increased formation of interstrand DNA cross-links in the nm23-H1 transfectants showing overexpression of nm23-H1, and there was no difference in the rates of DNA repair. Since it also was confirmed that the nm23-H1 level was increased in S phase (Caligo et al, 1995), and cisplatin has the strongest cytotoxic effect in S-G2 phase (Nguyen et al, 1993), one possibility is that nm23-H1 may play a role in cisplatin-induced cytotoxicity via modulating the cell cycle. However, our data with flow cytometry demonstrated that there was no difference in the cell cycle between parental YES-2 and YES-2/Neo and YES-2/AS-12 (data not shown). Recent studies have shown that mitochondria play an important role in the cytotoxicity or apoptosis induced by cisplatin, but not 5-FU or etoposide (Andrews and Albright, 1992; Olivero et al, 1995). The MTT assay is considered to be a representative mitochondrial function assay (Berridge and Tan, 1993); therefore, it is possible that nm23-H1 may be directly related to mitochondrial dysfunction induced by cisplatin in OSCC. This concept is supported by a previous report that respiration could be aided by ADP formed by adenylate kinase or nm23/NDP kinase in mitochondria (Gauthier et al, 1990). Further studies are required to clarify the roles of nm23-H1/NDP kinase A in mitochondrial dysfunction and/or apoptosis induced by cisplatin in human OSCC cells.

There were no differences in resistance to 5-FU between YES-2/AS-5 and YES-2/AS-29 and YES-2/Neo; however, YES-2/AS-12, with markedly reduced expression of nm23-H1 protein, showed increased resistance to 5-FU. The reason why only YES-2/AS-12 showed increased resistance to 5-FU remain unknown. It has been reported that several molecules such as Bcl-2 family proteins and dihydropyrimidine dehydrogenase contributed to the sensitivity to 5-FU (McLeod et al, 1998; Nita et al, 1998). Current study is attempting to clarify the relation or the interaction between nm23-H1 and these molecules in YES-2/AS-12.

In conclusion, the close relation of nm23-H1 status with resistance to cisplatin suggests that it may be a useful marker for

cisplatin-based chemotherapy in patients with OSCC. More interestingly, gamma linoleic acid, n-6 polyunsaturated fatty acid, was reported to increase the expression of nm23-H1 in human cancer cells (Jiang et al, 1998). Thus, modulating tumour nm23-H1 expression may be considered as a potential therapeutic strategy in combination with cisplatin treatment in OSCC patients.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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