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European Journal of Cancer 38 (2002) 1600–1606

European Journal of Cancer

www.ejconline.com

Differential expression of the suppressor PML and Ki-67 identifies three subtypes of human nasopharyngeal carcinoma

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Received 24 August 2001; received in revised form 14 December 2001; accepted 21 February 2002

Abstract

The promyelocytic leukaemia (*PML*) gene, which encodes a transformation and growth suppressor, was found to regulate transcription and apoptosis. *PML* was first identified at the chromosomal translocation break-points t(15;17) of acute promyelocytic leukaemia and the gene product may mediate cell-cycle control and apoptosis. PML was found to interact with the co-transactivator CREB binding protein (CBP) and the apoptotic-modulator Bax. To determine if PML, CBP and Bax may be involved in solid tumours, such as the nasopharyngeal carcinoma (NPC), a rare neoplasia that is prevalent in Southern China, the expression of these proteins and the proliferation marker Ki-67 was analysed by immunohistochemical staining. Expression of PML in the PML-oncogenic domain (POD) or nuclear bodies in most NPC was inversely correlated with the expression of Ki-67. In addition, based on PML expression patterns in NPC three subtypes could be identified, namely, Subtype-1, with strong PML expression in POD structures and with low Ki-67 staining; Subtype-2, where PML was expressed in a homogeneously diffused pattern, but with a low intensity in the tumour cells; while Ki-67 was expressed in a moderate number of cells and Subtype-3, where the majority of tumour cells were PML-negative, while a considerable number of tumour cells were strongly labelled with Ki-67. Furthermore, CBP was present in most of the NPC cells with moderate-strong nuclear staining, while the expression in non-tumour cells were relatively weak. However, there was no direct correlation between PML and CBP expression in the NPC examined. In addition, there was low or no expression of Bax in the NP and NPC. This is, to our knowledge, the first report describing PML and CBP expression in NPC and our data strongly suggests that PML and CBP, but not Bax, may play a role in the transformed phenotypes of NPC.
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Keywords: Suppressor; PML; Nasopharyngeal carcinoma; Bax; Ki-67; CBP

1. Introduction

Nasopharyngeal carcinoma (NPC) is a rare, but unique, neoplasm prevalent in Southern China observed in 25–50/100 000 in the Guangdong and Guangxi provinces as well as in Taiwan, but with a lower incidence among the northern Chinese (3/100 000) [1–4]. The Cantonese are the most frequently affected population and have an incidence rate nearly 100-fold higher than that seen in Caucasians [2–4]. The major risk factors for NPC have been identified which include the Epstein–Barr virus (EBV), environmental factors such as salt fish in infancy, and genetically-determined susceptibility [1–3]. Recent molecular studies indicate that activation of proto-oncogenes or transforming genes such as *LMP1*, *ras*, *c-myc* and *bcl-2* are often found in NPC [4]. In addition, inhibition of function and/or alterations have been identified in a number of tumour suppressor genes including *FHIT*, *p53, p16* and *p15* [2–4]. Although

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0959-8049/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0959-8049(02)00080-1

cytogenetic studies have indicated that chromosomes 3p, 9p, 11 and 14 are frequently altered in NPC, many of the genes responsible at these loci are still unknown [4]. Moreover, not all samples of NPC contain these mutations or aberrations, and it is believed that other suppressor genes or critical cellular-genes may also play a role in the complicated process of tumorigenesis [1–4].

We are interested in the *PML* gene which encodes a growth and transformation suppressor, and has been identified at the chromosomal translocation break-point t(15;17) of acute promyelocytic leukaemia (APL) [7–13]. This translocation event fuses the *PML* and the retinoic acid receptor-α (*RARA*) genes and encodes the PML-RARA and RARA-PML fusion proteins. PML is a nuclear phosphoprotein, which has been shown to inhibit cell growth and the transformed phenotypes of tumour cells [12]. PML has also been found to accumulate at the border of a nuclear structure, known as the nuclear body (NB) or PML oncogenic domain (POD) [7–12]. An earlier report has shown that PML may recruit the CREB binding protein (CBP) into the POD structure and could also enhance the transcriptional activity of the nuclear receptor [14,15]. Other reports have indicated that PML may interact with the apoptosis-modulator Bax [8,9,16]. PML also binds to several cellular proteins including Sp-100, NDP55, PIC 1/SUMO-1 and Int-6 and to viral proteins [17–21]. The POD may represent a large coactivation complex for transcription and PML is involved in cell cycle progression and viral replication [22–27]. A significant increase in PML nuclear bodies and a non-aggregated soluble form were found in cells progressing through the G1 and S phases of the cell cycle. PML is also a member of a novel family of ring-finger proteins that contain one or two other Cys/His clusters (B boxes) and a putative coiled-coil, in addition to the classical C3HC4 RING finger motif (RBCC configuration) [5–12]. The *PML* gene displays altered expression patterns in many types of cancers including the solid tumours [28–31]. Koken and colleagues [28], analysing various types of breast and colon neoplasms indicated that PML expression gradually increased in samples of benign dysplasia to carcinoma, and when the malignant cells turned invasive, they lost PML expression. There have been conflicting reports indicating increased or decreased levels of PML in different types of cancers [20–22,32–35]. For example, enhanced expression of PML was found in HCC, while in lymphoma, oat cell lung cancer, colon and breast cancers, PML levels were decreased [22,32]. Thus, the role of PML with regard to the proliferation and the transformed phenotypes of various neoplasias, especially rare tumours such as NPC, is still unclear.

We have previously reported on the cell-cycle regulation of DNA damage-induced expression of PML [33], and have identified a lack of expression of PML in small cell lung carcinoma [34] and altered expression of PML in hepatocellular carcinomas (HCC) and hepatitis tissues [32]. Here, we determined if the expression of PML, and two of the PML-associated proteins, CBP and Bax is altered in NPC samples from Hong Kong by immunohistochemical staining.

2. Patients and methods

2.1. Patients and samples

All of the samples were from Hong Kong Chinese patients treated at the Prince of Wales Hospital. Twenty two samples of NPC, which were diagnosed as undifferentiated tumours, were obtained. There were samples from 16 male (73%) and 6 female NPC patients (27%). Non-tumorigenic nasopharyngeal-tissues (NP) from 8 people with lymphoid inflammation or proliferative diseases served as controls.

2.2. Immunohistochemical staining

Immunohistochemical staining was performed with an affinity-purified rabbit antibody raised against the recombinant PML protein (1:2000) as previously reported in Refs. [32–34]. The proliferation marker Ki-67 was stained for using a mouse anti-human Ki-67 monoclonal antibody, which was provided as a ready to use reagent by Zymed Lab. (No. 08-0156). Antibodies against CBP (A-22, at 1:150 dilution) and Bax (1:150) were polyclonal IgG of rabbits from the Santa-Cruz Lab. The antibody for Bax was Bax (N-20), catalogue No. sc-493, which was a rabbit IgG with epitope for the N-terminal region of the human Bax protein.

In brief, samples obtained from resected tissues and tumours, were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin. Tissue sections were prepared in polylysine-coated slides and all steps were performed at room temperature. Slides were first heated at 60 °C in an oven. After dewaxing with xylene and rehydration with gradient alcohols, the slides were heated to boiling in 10 mM sodium citrate buffer pH 6.8 for 10 min in a microwave oven. After treatment with 3% hydrogen peroxide in methanol to inactivate the endogenous peroxidase activity, the samples were incubated with the blocking solution containing 5% bovine serum albumin (BSA) in PBS, for 20 min, and then reacted with the primary antibody for 1–2 h at room temperature. After washing 3–4 times with PBS, the samples were incubated 30 min each with the corresponding secondary antibodies (1:2000 dilution), goat anti-rabbit IgG- or anti-mouse IgG-conjugated with biotin, and then with avidin-conjugated with horseradish peroxidase or alkaline phosphatase (BioGenex Lab., San Ramon, CA, USA). The slides were washed with PBS after each step.

Table 1
Expression of PML, Ki-67, CBP and Bax in non-tumour nasopharyngeal tissues (NP)

NP samples	PML (%) (I)	Ki-67 (%) (I)	CBP (%) (I)	Bax (%)
1. NP	71±10 (+++)	0.2 ±0.1 (+)	55±10 (+)	0
2. NP	75±5 (++)	2±1 (++)		
3. NP	80±8 (+++)	3±1 (+++)	50±8 (+)	0
4. NP	90±5 (++)	5±2 (++)	55±10 (+)	0
5. NP	75±(8) (++)	1.2±0.2 (++)	60±8 (++)	
6. NP	81±7 (+++)	1.5±0.3 (+)		
7. NP	68±10 (+++)	9±2 (+++)		
8. NP	88±9 (++)	7±2 (+++)	60±10 (++)	

The intensity of staining is represented by (I), while the ratio of positive cells is represented as (%).

The slides were then developed with substrates for peroxidase or alkaline phosphatase (Histostain kit and Double labeling kit, Zymed Lab., S. San Francisco, CA, USA). After counterstaining with haematoxylin and briefly washing with 30 mM ammonium hydroxide, the slides were mounted with an aqueous or permanent mounting medium.

The expression of PML in the cells as a homogeneous staining or in the PML oncogenic domain (POD) or nuclear bodies, was rated as follows: ++++ and +++, very strong or >20 POD; ++, strong or 11–20 POD; +, moderate or 5–10 POD; ±, weak or partial, or 1–4 POD; and −, negative. The same scoring criteria were applied for the other antigens as well. For the proliferation marker Ki-67, the deparaffinised and rehydrated slides were digested with 0.1% trypsin in PBS for 10 min at room temperature for antigen retrieval. After washing with PBS, the samples were heated in the microwave and processed as described above. The incubation was for 1 h at room temperature. The slides were digested with 0.1% trypsin for 10 min at room temperature after deparaffinisation and rehydration as described above.

The samples were evaluated independently in 5–10 different regions of each slide for three separate experiments. The slides were analysed by three people separately who were blinded prior to the evaluation. The expression of each antigen was designated as the average of the percentage of cells stained positive together with the variance. The average intensity of staining (I) was also included as shown in Table 1. The antibody for Bax, was first tested with tissue samples from human breast ductal carcinoma, as a positive control. These samples showed strong reactivity. The PML and Ki-67 controls included HeLa, small cell lung carcinoma and fibroblast cell lines transfected with PML, as well as normal lung, adenocarcinoma and squamous cell carcinoma tissues, while the low expressing samples included small-cell lung carcinoma tissues and cell lines. Statistical analysis of the expression of PML and Ki-67, as well as the clinical parameters, were calculated using the Web Chi Square Calculator (http://www.georgetown.edu/cball/webtools/web_chi.html) and the Student *t*-test. The relative expression levels were calculated by multiplying the percentage of cells stained positive with the intensity of the stainings.

3. Results

3.1. Expression of PML and Ki-67 proteins in human non-tumorigenic nasopharyngeal tissues

Immunohistochemical analysis of PML and Ki-67 protein expressions using double-staining was performed in 8 cases of non-tumorigenic nasopharyngeal tissues (NP), all showed hyperplasia, and in 22 cases of NPC, which were all classified as undifferentiated carcinomas. The data were summarised from three independent experiments and the cases were randomly selected in each experiment. The data were expressed as the average of the percentage (%) of cells stained positive in each sample together with the variance and I. The non-tumour tissues in the NPC samples served as internal controls. The results demonstrated that the expression of the PML protein in the tumour cells of NPC were variable (Table 2) when compared with the epithelial cells in the NP samples.

In the NP samples (Fig. 1, Panels a and b), all of the samples analysed (8/8) showed strong or very strong PML expression. On average, 77% of the epithelial cells showed PML positivity with the intensity ranging from strong (++) to very strong (+++) staining. In addition, more than 95% of the endothelial cells in the NP tissues showed PML expression with very strong (+++) staining, while the lymphocytes in the stroma showed weak to moderate (± to +) staining. For the epithelial cells, which are considered to be the precursor cells of NPC, 53% were found to express PML in the POD (PML oncogenic domain) or nuclear bodies (NB), while 24% were found to be homogeneously staining for the whole nucleus (range 12∼50%). Double staining of the proliferation marker Ki-67 together with PML showed that most of the cells stained positive with PML were Ki-67-negative and vice versa. Four per cent of the epithelial cells from NP were Ki-67-positive and 2% of them co-expressed with PML. In 5 of the cases, the germinal centres could be clearly identified which contained a high percentage of Ki-67-positive lymphocytes, ranging from 29 to 62% of the total cells, some of which co-expressed with PML (data not shown).

3.2. Expression of PML and Ki-67 proteins in human nasopharyngeal carcinoma tissues

In the NPC samples, double-labelling experiments (Fig. 1, Panels a, c, e and g) showed that PML and Ki-67

protein expressions in the NPC samples were variable, and inversely correlated. Twelve of the 22 samples contained a high percentage of tumour cells with PML expression (ranging from 70 to 90% of the total cells) and with an intensity ranging from moderate (+) to very strong (+++). Based on the PML and Ki-67 staining patterns in the tumour cells of NPC, three Subtypes of NPC could be categorised, namely Subtypes-I, -II and-III (Table 2). 8 out of 22 cases (36%) were Subtype-I, in which PML expression in this group was similar to those of the epithelial cells in the NP samples, namely high PML and low Ki-67 expression (Fig. 1, Panel c). A high percentage of tumour cells showed POD/nucleoplasm staining (POD-positive) with

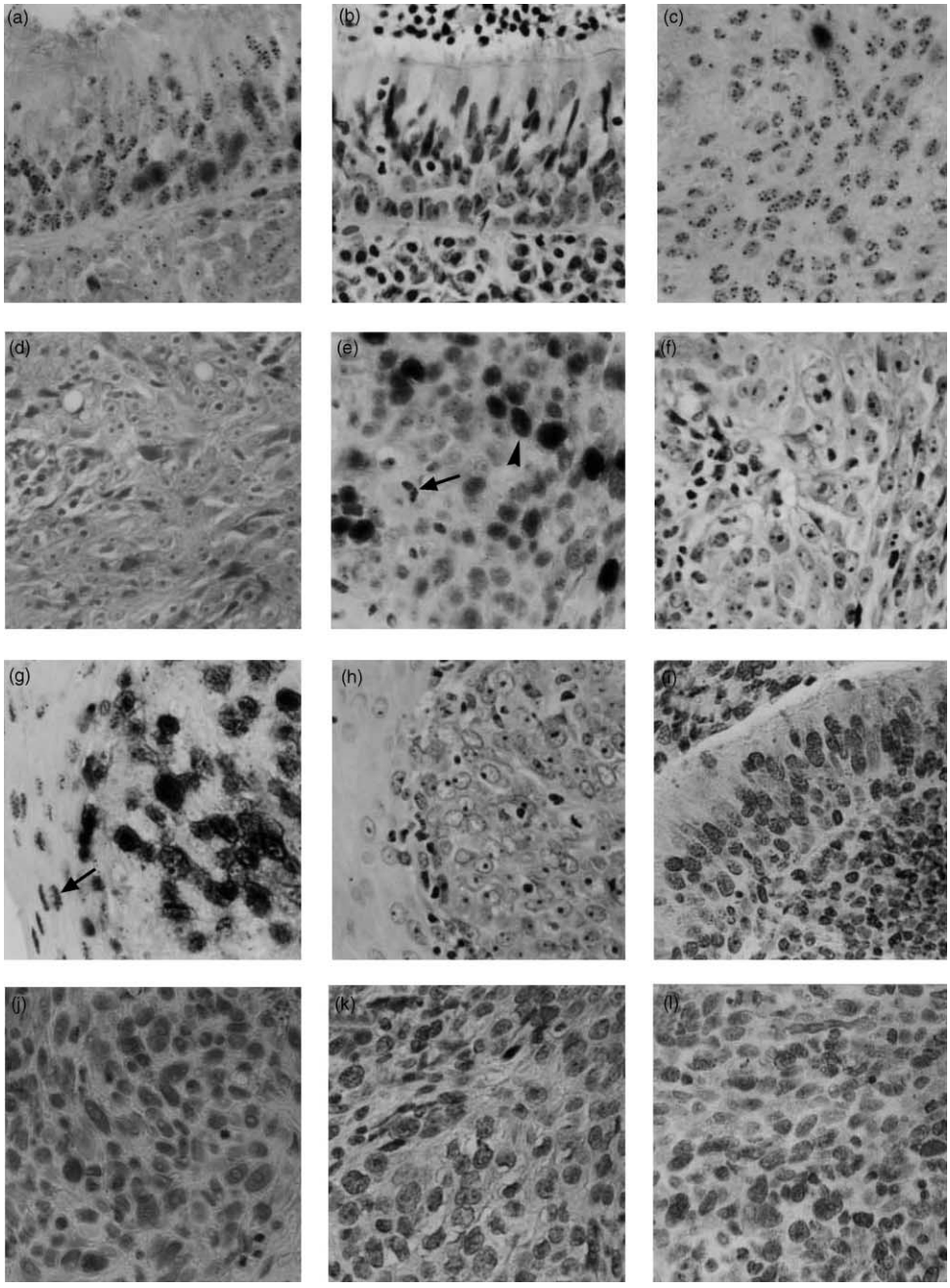


Fig. 1. Expression of PML, Ki-67 and CBP in non-tumour nasopharyngeal tissues (NP) and Nasopharyngeal carcinoma (NPC) tissues by immunohistochemical staining. Panels a, c, e and g: expression of PML and Ki-67. Paraffin sections of NPC and NP tissues were immunohistochemically stained with antibodies against PML and Ki-67. Panels a, c, e and g are samples of NP, NPC Subtypes-I, -II and -III, respectively. Panels b, d, f and h: haematoxylin and eosin staining. NP and NPC Subtypes-I, -II and -III of the corresponding samples in Panels a, c, e and g were stained with haematoxylin and eosin, respectively. Panels i, j, k and l: expression of CBP. Samples of NP and NPC Subtypes- I, -II and -III, respectively, were immunohistochemically stained with antibody against the PML-modulating protein CBP.

Table 2
Expression of PML, Ki-67, CBP and Bax in nasopharyngeal carcinoma (NPC)

NPC samples	PML (%) (I)	Ki-67 (%) (I)	CBP (%) (I)	Bax (%)
Subtype-I				
1. NPC	80±7 (++)	19±8 (++)	83±9 (++)	
2. NPC	76±10 (+++)	18±10 (++)	76±8 (+)	0
3. NPC	86±8 (++)	20±7 (++)		
4. NPC	80±7 (+++)	10±5 (++)		
5. NPC	90±5 (+++)	1±0.2 (+)	72±10 (+)	0
6. NPC	80±9 (+++)	1±0.2 (++)	66±8 (+)	0
7. NPC	81±8 (+)	1±0.3 (++)		
8. NPC	85±10 (++)	12±6 (++)		
Subtype-II				
9. NPC	92±5 (++)	5±2 (+)	90±5 (+)	0
10. NPC	85±6 (++)	4±1 (++)	89±6 (+)	0
11. NPC	70±8 (+)	30±8 (++)		
12. NPC	60±10 (+)	27±10 (+)	59±10 (++)	
13. NPC	90±5 (++)	20±8 (++)	64±12 (+)	
Subtype-III				
14. NPC	27±10 (++)	30±10 (++∼+++)	88±8 (+)	
15. NPC	11±5 (++)	20±9 (++∼+++)	58±15 (+)	0
16. NPC	12±6 (+)	31±10 (++∼+++)	91±4 (+)	0
17. NPC	12±5 (+)	23±8 (++∼+++)	54±10 (+)	
18. NPC	40±11 (+)	31±10 (++)		
19. NPC	35±8 (+)	24±8 (++)		
20. NPC	26±10 (++)	25±12 (++∼+++)	68±8 (+)	0
21. NPC	31±10 (++)	19±8 (++∼+++)	93±3 (++)	
22. NPC	18±8 (++)	23±6 (++)	88±4 (+)	

The intensity of staining is represented by (I), while the average of ratio of positive cells is represented as (%) with the variance included.

an average of 68% (range 50∼90%); and 14% (range 5∼37%) as nucleoplasmic staining (homogeneous staining). The staining intensity ranged from moderate (+) to very strong (+++). The expression of the proliferation marker Ki-67 in this group was low, range 1∼20%, but the percentage of Ki-67-positive cells that co-expressed with PML was higher than those in the NP samples, at 12%.

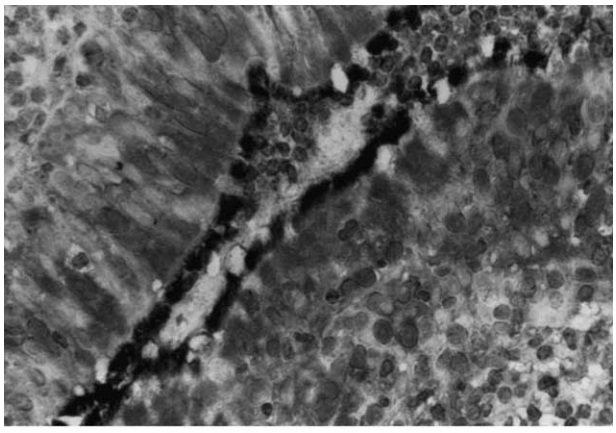
In Subtype-II (5/22 cases, i.e. 23%), double-staining with PML and Ki-67 (Fig. 1, Panel e) showed moderate expression of both PML and Ki-67. The percentage of POD-positive tumour cells decreased to 8% (range 1.3–17%) with a decreased intensity level from + to ++ compared with Subtype-I. The homogeneously stained cells increased to 57% (range 27∼91%) with an intensity level ranging from + to ++ (Table 2). However, the expression of Ki-67 in this group was higher than the first group, namely 4∼30%. The co-expression rate of Ki-67 and PML was 11% in the tumour cells (range 2∼18%).

The Subtype-III samples, which consisted of 41% (9/22 cases) of the total NPC examined, showed low PML and high Ki-67 expression (Fig. 1, Panel g). PML expression in the tumour cells was significantly decreased for both the POD-positive and homogeneous-staining patterns, as well as for the percentage of cells and the intensity of staining. The average numbers of POD-positive cells was 4% (range 1∼13%) and 9% with homogeneous-staining (range 2–21%). The intensity of staining for PML ranged from moderate (+) to strong (++). However, the Ki-67-positive tumour cells in this group increased significantly, the numbers of tumour cells expressing Ki-67 ranged from 19 to 31% with an increased intensity of strong (++) to very strong (+++); and only 6% of them (range 1.5–10%) co-expressed with PML. In contrast, the internal control of the non-tumour tissues of this group of samples, showed PML and Ki-67 expression similar to the NP samples. It was also observed that the tumour cells in this group were very compact and varied in size. Statistical analysis of the expression of PML and Ki-67 was then done using the Web Chi Square Calculator (http://www.georgetown.edu/cball/webtools/web_chi.html). The results were: Chi-square = 1236, while the P value was less than or equal to 0.001. The distribution was found to be significant.

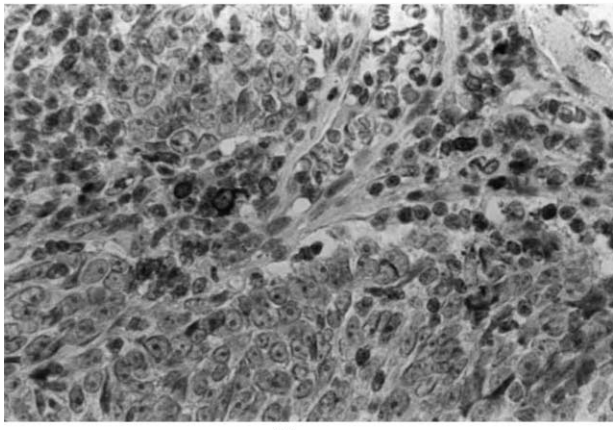
The expression of PML was then correlated with some of the clinical parameters of NPC. Subtype-I contained three T1, three T2 and two T3, while Subtype-II contained one T1, two T2 and three T3. Subtype-III contained two T1, four T2 and three T3. The Chi-square for the pathological correlation was 1.4, while the P value was less than or equal to one. The distribution is considered not significant. In addition, the 30 month survival rate for Subtypes-I and -II was 71% (9/13), while that for Subtype-III was 76% (6/9), which was not significantly different.

3.3. Expression of CBP and Bax, the presumptive partners of PML, in human NP and NPC

It has been previously reported that the suppressor PML may bind to a transcriptional co-activator CBP, as well as to Bax, a member of the Bcl-2 apoptotic/anti-apoptotic gene family. The interaction of PML and CBP could induce transcriptional activation of target genes, while PML and Bax expression could modulate apoptosis. To determine if CBP may play a role in NPC cells, we analysed the expression of CBP and Bax by immunohistochemical staining. As shown in Fig. 1, Panels i, j, k and l for NP and NPC Subtypes-I, -II and -III, respectively, the expression of CBP in NP was moderate in the epithelial cells, while low expression was found in the stromal cells. In the NPC tissues, strong expression was found mostly in the tumour cells in the stromal regions. Unlike Ki-67 and PML, there was no correlation in the expression of PML and CBP in the various Subtypes of NPC. Similarly, there was no correlation in the expression of PML and Bax. Bax was not expressed in NP nor in any of the NPC samples examined (Fig. 2, Panel a for NP and Panel b for NPC), while considerable expression was found in breast cancer tissues, which served as positive controls (data not shown).



(a)



(b)

Fig. 2. Expression of Bax in non-tumour nasopharyngeal tissues (NP) and nasopharyngeal carcinoma (NPC) tissues by immunohistochemical staining. Samples of NP (Panel a) and NPC (Panel b) were immunohistochemically stained with antibody against Bax.

4. Discussion

In this report, we were able to categorise three Subtypes of NPC based on the expression of PML and Ki-67. The expression of PML was inversely correlated with that of the proliferation marker Ki-67 in these Subtypes. PML expression in Subtype-III was found to be either very low or undetectable in all 9 samples examined, while Ki-67 expression was high. In contrast, considerable expression of PML was found in Subtypes-I and -II, with relatively low Ki-67 expression. These data suggest that PML could be a useful biomarker for NPC, and could be another growth suppressor that plays an important role in the oncogenesis of NPC. These data are also in accordance with the findings of Koken and colleagues [28] indicating that decreased PML expression was associated with more aggressive tumours. Our results may also account for the conflicting reports indicating increased or decreased levels of PML in different types of cancers. On the other hand, the differential expression of PML protein could be a reflection of the number of cells in the different phases of the cell cycle. The Subtype-III NPC, which were apparently more active in cell growth and therefore stained more strongly positive with the proliferative marker Ki-67, showed negative or low levels of PML. In contrast, the neighbouring non-tumour cells, which were mostly non-proliferative and stained negative for Ki-67, showed strong PML expression. These results were in accordance with previous reports supporting the notion that PML is a growth suppressor. However, we failed to observe a correlation between the clinical outcome of the patients and the Subtypes of NPC. An earlier publication by Masuda and co-workers also indicated that there was no correlation between the expression of Ki-67 and clinical outcome in NPC, indirectly corroborating our findings [36]. Cancer development, including that of NPC, appears to be a multistage-multihit process in which normal cells progress to malignancy through an accumulation of numerous genetic and epigenetic alterations [1–6]. The complexity of tumour progression and the diversity in treatment and response of patients may have obscured the relationship between the biology of the tumours and these molecular markers. In addition, many of these Chinese patients could also be treated with Chinese traditional medicine or alternative therapies which could obscure any correlations. Another explanation is that the sample size of patients was too small and, thus, a larger cohort of analysis might be warranted in the future.

It is also unclear if the decrease of PML expression in the Subtype-III of NPC is the consequence of mutation, transcriptional suppression or modulation of *PML* at the protein level. Altered stability or the escape of detection of the PML protein as it is being modified or sequestered by cellular proteins cannot be ruled out. PML co-localises with several cellular proteins including CBP and Bax [25–28]. PML could be modulated by these binding proteins or vice versa, which could then induce changes in nuclear processes including transcription, cell-cycle control and apoptosis. It was postulated earlier that the nuclear bodies or POD might represent storage sites of certain matrix proteins readily accessible throughout the chromatin in response to stress or other effectors that induce global nuclear changes [28]. Thus, PML might act as a matchmaker to recruit or sequester other proteins in or out of the POD. However, the expression of CBP and Bax did not show any correlation with that of PML in NPC. CBP expression was found to be moderately upregulated in most of the NPC examined, while Bax was not expressed at all in these NPC and NP. The levels of free CBP in the cells could have blocked the PML-associated CBP, whilst Bax may not play a role in the phenotypic expression of NPC. In conclusion, to our knowledge, this is the first report on PML expression in NPC and we have identified three Subtypes of NPC based on the expression levels of PML and Ki-67.

Acknowledgements

This research was supported, in part, by CUHK Grants 26809335, 20404940, 2040653 and 2040614, Hong Kong RGC Earmarked Grant CUHK4260/97M (S.F. Leung) and CUHK4280/98M (J.Y.H. Chan), and a Visiting Associate Professorship, National Science Council, Taiwan, to J.Y.H. Chan.

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