

RESEARCH ARTICLE

Epidermal growth factor receptor (EGFR) abundance correlates with p53 and Bcl-2 accumulation and patient age in a small cohort of North African nasopharyngeal carcinoma patients

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ABSTRACT. In North Africa, nasopharyngeal carcinoma (NPC) is characterized by a bimodal distribution involving a juvenile (≤ 30 years old) and an elder population (>30 years old). The Epstein Barr virus oncogene LMP1, the anti-apoptotic Bcl-2 protein and the tumor suppressor p53 have recently emerged as biomarkers of the disease. EGFR/Erbb1 expression is detected in the majority of NPC tumors with advanced disease. To obtain greater insight into the potential oncogenic mechanisms specific to these two NPC populations, we examined the correlation between EGFR expression and patient age, and determined the molecular profiles of its associations with the biomarkers of NPC. We performed an immunohistochemical analysis of the latter molecules in NPC specimens from eleven Algerian patients (six patients ≤ 30 years of age and five patients >30 years of age) using the LSAB method. Evaluation of the biopsies, based on the intensity of staining and the percentage of positive cells, showed that LMP1 expression was higher in patients under 30 years of age. Conversely, EGFR, like Bcl-2 and p53, was significantly up-regulated in tumors from elderly patients. Analysis of all tumors showed that EGFR expression was constantly (100%) associated with high p53 nuclear accumulation and Bcl-2 expression in LMP1-positive tissues. Biopsies negative for Bcl-2 staining were found to display low amounts of p53 (100%), and to be constantly negative for EGFR (100%). Molecular classification of all NPC tissues showed that the majority of patients displaying a EGFR+/LMP1+/Bcl-2+/p53-high molecular pattern were in the older age group. On the other hand, the most of the EGFR negative results were associated with the juvenile form of the disease and were characterized by an important diversity of molecular patterns. Our preliminary results suggest that in Algerian patients, the bimodal distribution of NPC might be related to distinct expression profiles of viral and cellular biomarkers of NPC.

Keywords: NPC, Epstein-Barr virus, EGFR, LMP1, p53, Bcl-2

Nasopharyngeal carcinoma (NPC) results from a malignant process affecting epithelial cells of the nasopharynx. It is rarely found in developed countries, however, there is a high incidence of this condition in South East China. In North Africa (Algeria, Tunisia and Morocco) the incidence is intermediate. In Algeria, it is the most common cancer affecting the ORL area, and, as such, poses a serious public health problem. It has an annual incidence of three to eight cases per 100,000 inhabitants. Men are twice as likely to develop NPC as women. The undifferentiated nasopharyngeal carcinomas (UCNT/WHO class III) that constitute the vast majority of NPC in North Africa are consistently associated with a latent Epstein-Barr virus (EBV) infection [1].

Genetic and environmental factors such as human leukocyte antigen (HLA) haplotypes [2], and consumption of food with volatile nitrosamines (rancid fat) are thought to predispose to NPC [3, 4]. In North Africa, the distribution of NPC according to patient age was reported to be bimodal, with a large group of patients being around 50 years old (80%), and a smaller group between 10 and 30 years old (20%) [5]. In Algeria, a reduction of the incidence of NPC in the juvenile group has been observed during the last decade [6].

Recent studies have shown a particular interest in the mechanisms of transformation of nasopharyngeal cells in presence of the virus. Depending on the expression

of up to nine EBV-encoded proteins, three types of latency-associated genetic programs can be displayed by EBV-transformed cells. The latency program type III, found in the UCNT, is consistently associated with expression of the viral oncogenic latency membrane protein (LMP1). LMP1 is thought to induce cell transformation through a constitutive activation of several transcription factors, such as NF- κ B, AP1 and Stat-3 [7, 8], which mediate cell proliferation, resistance to apoptosis, immortalization, tumor invasion and metastasis [9]. Recent investigations have proposed p53, Bcl-2 and EGFR/Erbb1 as biomarkers that could contribute to NPC development, based on the following observations: 1) p53 accumulation is observed in the majority of NPC [10]; 2) expression of the anti-apoptotic Bcl-2 protein has been reported to be up-regulated, *in vitro*, by LMP1 in epithelial cells [11]; 3) EGFR is often associated with epithelial cell transformation and advanced stages of head and neck tumors [12, 13]. The tumor suppressor p53 protein and Bcl-2 have been found to be more abundantly expressed in elderly patients [5, 14], while LMP1 has been detected at higher levels in NPC specimens from juvenile patients in Tunisia [15]. However, to date, no previous studies have reported any difference in EGFR levels or any correlation between expression of this molecule and LMP1, p53 or Bcl-2 levels, in either the juvenile or the elderly group. Thus, we were interested in, comparing the level of expression of these biomarkers in 11 tumors obtained from Algerian patients, representative of the bimodal distribution characterizing NPC in Algeria.

DONORS AND METHODS

Patients and tumor specimens

Primary NPC biopsy samples were collected, prior to any treatment, from 11 patients, at the Centre Pierre et Marie Curie, Mustapha Bacha Hospital in Algiers, between 2005 and 2009. The patients ages ranged from 13 to 60 years old (mean age: 34.4 ± 17.6 years). Six (54.5%) patients were less than thirty years old. The histological type of NPC was determined on tissue sections in accordance with the World Health Organisation (WHO) classification. Based on morphological examination, all tissues were confirmed as belonging to the group of undifferentiated carcinomas following hematoxylin and eosin staining (UC, WHO type 3). Patients had advanced disease (stage III and IV) at the time of evaluation (90%). All participants gave their informed consent for the present study, which was carried out according to the guidelines of the local Ethics Working Group.

Immunostaining

Sections from tumors from 11 NPC patients were stained with anti-LMP1, EGFR, p53 or Bcl-2 monoclonal antibodies. Three μ m sections attached to silanized slides were de-waxed in xylene and rehydrated in graded ethanol. They were then incubated for 45 to 60 minutes with the anti-LMP1 (CS1-4), Bcl-2 (Clone 124), p53 (Clone DO-07) or EGFR (Clone H11) antibodies (0.5 to 1 μ g/mL). For the EGFR antibody, the staining was preceded by a de-masking treatment: 5 min proteinase K (S3020; Dako) and 30 min retrieval solution (S1700; Dako) at 97°C. Primary antibody binding was visualized with biotin-labelled sec-

ondary antibodies and a streptavidin-peroxidase complex using diaminobenzidine (DAB) as a chromogenic substrate (LSAB-2 system, Dako).

Scoring method

Immuno-staining was scored on the basis of the percentage of positive tumor cells and the relative immunostaining intensity as previously reported [15]. Four consecutive microscope fields were analyzed. When the tissue had no staining, it was scored 0; if the tumor section revealed the presence of occasional positive cells (but not exceeding 25%), the section was scored 1; 2 when 26 to 50% cells were positive; 3 for 51 to 75% positivity and 4 for 76 to 100% staining. Immunostaining intensity was rated 0 for none, 1 (+) for weak, 2 (++) for moderate and 3 (+++) for intense. When the staining intensity was heterogeneous, each component of the tumor was scored independently and the results were summed up as follow: if a specimen contained 50% of tumor cells with moderate intensity ($2 \times 2 = 4$), 25% of tumor cells with intense immunostaining ($1 \times 3 = 3$), and 25% of cells with weak intensity ($1 \times 1 = 1$), the score was $4 + 3 + 1 = 8$. The maximal possible score was 12 [15]. The tumor sections were read and scored independently by two investigators.

Statistics

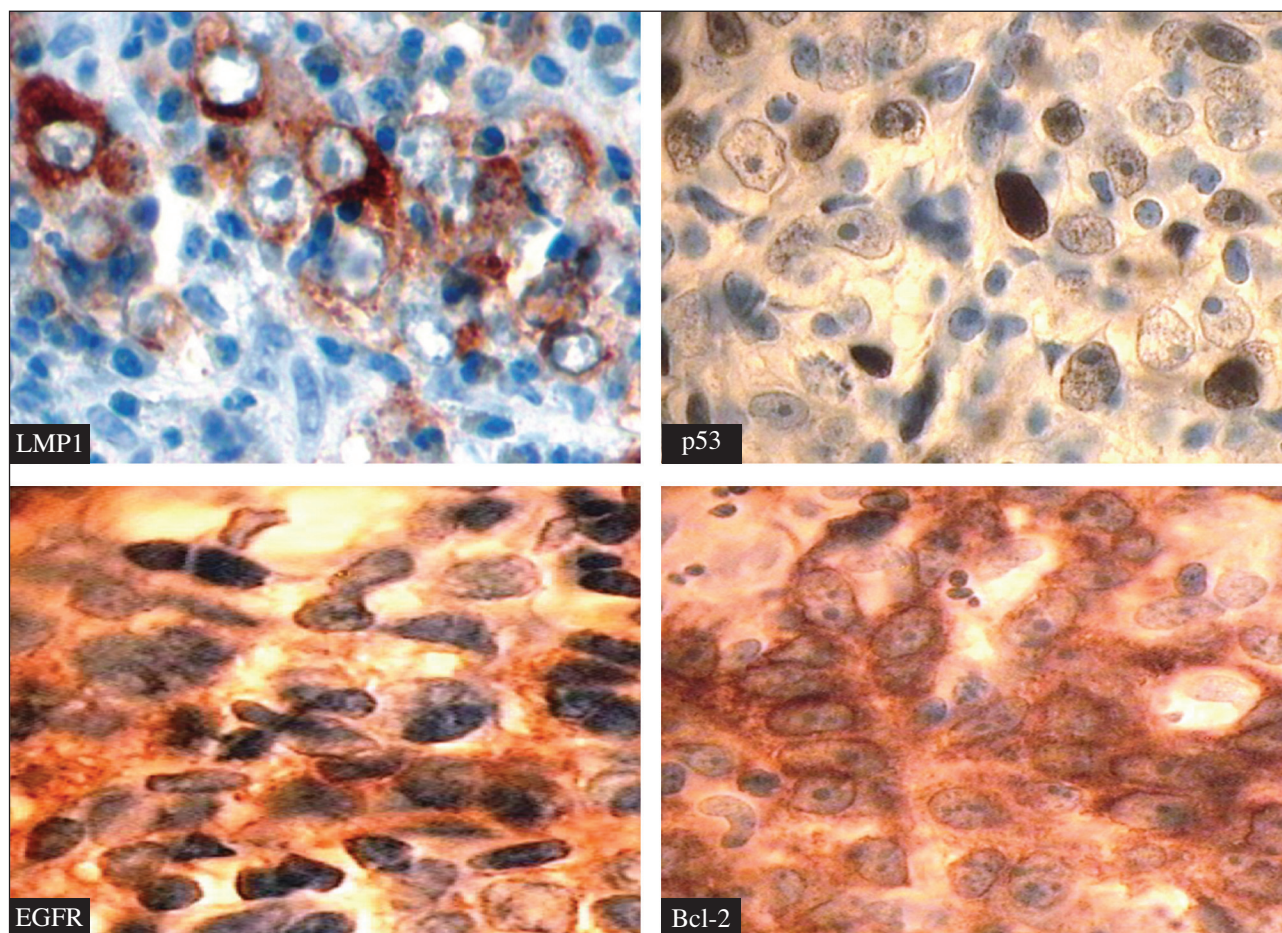
All results were expressed as mean \pm SD (standard deviation). Data analysis was performed using the statistical software. Student's *t* test was used for comparison between different groups.

RESULTS

NPC tumors of elderly patients are more often associated with EGFR positivity compared to NPC tumors of juvenile patients

In the recent years, a great deal of attention has been given to the analysis of p53, Bcl-2, EGFR and LMP1 during NPC progression [5, 14, 16]. The detection of some of these biomarkers was associated with poor prognosis and resistance to treatment [13, 17]. No report has hitherto revealed differences in the expression of these molecules between the juvenile form of NPC and the adult form in Algeria. In order to examine this further, eleven ($n=11$) tumors from patients were examined by immunohistochemistry (figure 1, table 1). Tissues analysis showed highly heterogeneous staining between tumors from different patients. Evaluation of the percentage of positive tumor cells revealed that, although all tumors were positive for p53 staining, only 45% were as scored 3+ (High); 55% were scored 1+ and 2+ (Low). Half of the specimens tested were negative for LMP1, 55% were positive for EGFR, and 73% were positive for Bcl-2 (table 2).

Analysis of the mean age for the detection of each biomarker showed that tumors with low amounts of p53 were found in younger patients (mean age 28.2 ± 15.3) compared to those with high amounts of p53 (39.5 ± 19.2 ; $p=0.31$). This observation was also true for Bcl-2 (20.7 ± 8.6 (-) *versus* 39.5 ± 17.7 (+) $p=0.12$) and EGFR

**Figure 1**

Immunohistochemical analysis of UCNT tissues. NPC biomarkers immunoreactivity was respectively located at the membrane and the cytoplasmic level for LMP1, in the nuclei for p53, at the membrane level for EGFR and in the cytoplasm for Bcl-2. (100x). The sections were read and scored independently by two investigators.

(28.2 ± 15.3 (-) *versus* 39.5 ± 19.2 (+) $p = 0.31$). Patients' mean ages for tumor specimens with detectable and non-detectable LMP1 were respectively, 32.4 ± 19.1 (-) and 36 ± 18.1 (+) $p = 0.75$ (table 2). These results suggest that EGFR, together with p53 and Bcl-2, was more often up-regulated in tumors from elderly NPC patients than in those from juvenile patients.

EGFR is significantly less expressed in juvenile NPC tumors than in NPC tumors from elderly patients

Epidermal growth factor receptor (EGFR) over-expression has been proven to be an independent predictor of poor clinical outcome in NPC [13, 18]. Moreover, the oncogenic viral protein LMP1 has been reported to be able, *in-vitro*,

Table 1
Individual immunohistological profiles of 11 algerian NPC patients.

Age (years)	LMP1	EGFR	p53	Bcl-2	Gender	Histological type
13	0	0	Low	0	M	UC, WHO type 3
15	0	1	High	2	M	UC, WHO type 3
16	3	1	High	2	M	UC, WHO type 3
19	3	0	Low	0	F	UC, WHO type 3
26	2	0	Low	3	F	UC, WHO type 3
30	0	0	Low	0	M	UC, WHO type 3
46	3	3	High	2	F	UC, WHO type 3
49	3	3	High	3	F	UC, WHO type 3
51	0	1	High	1	F	UC, WHO type 3
53	0	0	Low	2	M	UC, WHO type 3
60	1	3	High	3	M	UC, WHO type 3

11 NPC Tumors were classified following immunoreactivity, age, gender, and the histological type. Patients had advanced disease (stages III and IV) at the time of evaluation. Immunostaining intensity was rated 0 for none, 1 (+) for weak, 2 (++) for moderate and 3 (+++) for intense. p53 staining was classified as low reactivity (+ or ++ and high reactivity (+++).

Table 2
Comparison of patient's mean age per biomarker analyzed in 11 UCNTs.

	LMP-	LMP+	EGFR-	EGFR+	Bcl2-	Bcl2+	p53 low	p53 High
% cases	45	55	45,5	54,5	27,27	72,73	45,5	54,5
Mean age (years)	32,4	36	28,2	39,5	20,7	39,5	28,2	39,5
SD	19,1	18,1	15,3	19,2	8,6	17,7	15,3	19,2
p value	0,75		0,31		0,12		0,31	

Tumors were classified following immunoreactivity. Stainings were classified as either positive or negative for LMP1, EGFR, and Bcl2. p53 staining was classified as low reactivity (+ or ++) or high reactivity (+++). Values are given as mean age (years) \pm standard deviation (sd).

to up-regulate EGFR expression levels [19]. In clinics, evaluation of EGFR expression levels by immunohistochemistry, prior to EGFR-targeted therapy, has become important because of the detection of relatively significant rates of EGFR-negative tumors. In the Algerian population, this rate is estimated to be approximately 20% of all NPC tumors [20].

As LMP1 was reported to be differently expressed in juvenile and elderly patients, we wished to determine whether EGFR expression would follow a different expression pattern in these two populations. For this purpose, we compared the levels of EGFR expression in all NPC specimens using the LSAB method. Bcl-2 and p53 expression rates were used as controls because these markers have been previously reported to have specific patterns associated with the bimodal distribution of NPC [5, 14].

Highly heterogeneous staining was observed for all markers examined, between patients and within the tumor tissue for each single patient (table 1). True variations in the amount of each staining were visible in malignant cells from one patient to another, and within the same analyzed sample. Therefore, we evaluated the tissues examined using a scoring system based on the percentage of positive cells and the intensity of staining. We found that the rates of LMP1 detected by the CS1-4 antibody were higher in the juvenile specimens. We also observed that EGFR-expression scores followed an inverse pattern similar to the one observed for our controls, namely Bcl-2 and p53. Indeed, as high scores of EGFR were found in tumors of patients over 30 years of age, significantly lower scores were detected in tumors of juvenile patients (figure 2). These results suggest that EGFR, when expressed, is found at lower levels in the juvenile NPC patients compared to elderly NPC patients.

EGFR expression correlates with high p53 nuclear accumulation and Bcl-2 expression in LMP1-positive tumors

We next sought to determine whether distinct association profiles could be found between the NPC biomarkers studied and whether any molecular pattern could be over-represented in either the juvenile or the elderly patient categories. For this purpose, we first evaluated the percentages of cross-positivity of LMP1, EGFR, Bcl-2 and p53 for all tumors. To our surprise, we observed that in all specimens, EGFR expression correlated perfectly with the accumulation of high amounts of p53 (100% of cases), and Bcl-2 (100% of cases) (table 3). A partial correlation was found between EGFR and LMP1 (67% of cases). Conversely, tumors with no detectable EGFR consistently displayed low amounts of p53 (100% of cases,) and preferentially displayed non-detectable Bcl-2 (60% of cases) or LMP1 (60% of cases) (table 3).

Interestingly, we also observed that all Bcl-2-negative tumors were negative for EGFR (100% of cases), had low amounts of p53 (100% of cases), and were predominantly negative for LMP1 (67% of cases). In comparison, Bcl-2-accumulating tumors were associated with high positivities for EGFR (75% of cases), LMP1 (63% of cases), and displayed large amounts of p53 (75% of cases) (table 3).

Our observations indicate that EGFR expression is often associated with detectable LMP1, and is consistently associated with a high accumulation of p53 and Bcl-2. Conversely, the absence of EGFR expression correlated with low p53 accumulation and only slight expression of Bcl-2 and LMP1.

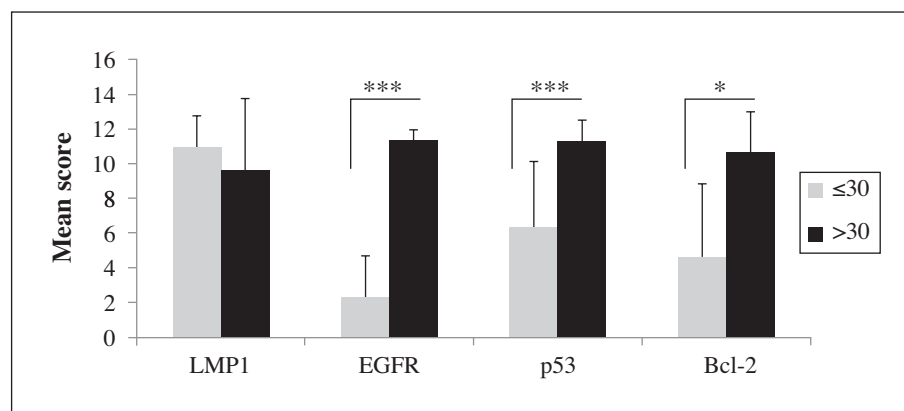


Figure 2

Semi quantitative analysis of UCNT specimens. Tumors from juvenile and elderly patients were analyzed using a scoring system based on the percentage of positive cells and the intensity of staining. Mean scores from LMP1 positive tumors (3 juveniles and 3 adults) were analyzed.

Table 3
Cross-evaluation of the expression of NPC biomarkers in 11 biopsies.

	EGFR-(n=5)	EGFR+(n=6)	LMP1-(n=5)	LMP1+(n=6)	p53 Low(n=5)	p53 High(n=6)	BCL2-(n=3)	BCL2+(n=8)
LMP1 +	40	67	-	-	60	67	33	63
LMP1-	60	33	-	-	40	33	67	38
p53Low	<u>100</u>	0	60	33	-	-	<u>100</u>	25
p53High	0	<u>100</u>	40	67	-	-	0	75
BCL2-	60	0	40	17	60	0	-	-
BCL2+	40	<u>100</u>	60	83	40	<u>100</u>	-	-
EGFR-	-	-	40	33	<u>100</u>	0	<u>100</u>	25
EGFR +	-	-	60	67	0	<u>100</u>	0	75

Values are expressed as percentages (%) of cases.

Major NPC tumors from EGFR+ elderly patients display an LMP1+/Bcl-2+/p53-high molecular pattern

To determine whether the previous results could be representative of specific molecular patterns present in NPC tumors from the juvenile and elderly patients, we compared the immunohistological profiles found in the tested tumors with patients' ages. We observed that the majority (3/4; 75%) of the samples obtained from elderly EGFR+ patients had an LMP1+/Bcl-2+/p53-high molecular signature. Conversely, two out of four EGFR- samples (50%) detected in the juvenile group, displayed an LMP-/Bcl-2-/p53-low molecular pattern. In addition, we observed that tumors from the juvenile group were characterized by a wide diversity of molecular patterns within the EGFR+ or EGFR- sub-groups (table 4). These results suggest that juvenile and the elderly patients preferentially present distinct, molecular signatures.

DISCUSSION

In this study, we examined the expression of EGFR, together with that of other cellular and viral biomarkers, in eleven Algerian NPC patients. We also compared the pattern of expression of these biomarkers with patient age. Our results show that EGFR was more often expressed in elderly patients. Tumor specimen evaluation showed a trend for EGFR to be expressed at low levels in juveniles.

Table 4
Nasopharyngeal carcinoma (NPC) molecular patterns observed in 11 patients.

EGFR+	Juvenile	Elderly
LMP1+/Bcl2+/p53High	1	3
LMP1-/Bcl2+/p53High	1	1
EGFR -		
LMP1+/Bcl2+/p53Low	1	-
LMP1+/Bcl2-/p53Low	1	-
LMP1-/Bcl2+/p53Low	-	1
LMP1-/Bcl2-/p53Low	2	-

Tumors from juvenile and elderly patients were analyzed following the expression of EGFR. Tumors were classified following the distinct molecular patterns observed by immunohistochemistry.

In elderly patients, EGFR expression was found to be often associated with detectable LMP1, and to be consistently associated with a high accumulation of p53 and Bcl-2. Conversely, the absence of EGFR expression correlated with low p53 accumulation and only slight expression of Bcl-2 and LMP1. The comparison of all tumors indicated that elderly patients preferentially present an EGFR+/LMP1+/Bcl-2+/p53-high molecular pattern. No particular signature was observed in tumors from juvenile patients.

Our results show that p53 and Bcl-2 products are less expressed in juvenile NPC. This observation is in agreement with a study conducted involving Tunisian patients [5, 14]. This suggests that Algerian and Tunisian NPC patients might share similar features with regards to the expression of these cellular biomarkers; this similarity was also true for LMP1. Moreover, the level of detection of LMP1 in our cohort (55%) was in the range of that observed in Asian studies (60%) [21, 22]. The difference might be due to the small size of our cohort, to the sensitivity of the detection method, and/or the clone of the antibody used for the LMP1 screening [15, 23].

We observed that the mean age of the patients with detectable and non-detectable LMP1 products was similar, but a semi-quantitative analysis of the biopsies revealed that the viral oncogene might be more expressed in juvenile patients. This analysis extended to cellular biomarkers of NPC, revealed that, as for p53 and Bcl-2 [5, 14], EGFR was also be present at low levels in NPC tumors of patients under the age of 30. Several studies have suggested that EGFR could be a major prognostic factor in head and neck cancer [24, 25], consequently highlighting it as a prime target for anticancer therapy. A significant accumulation of EGFR is associated with a greater efficacy of anti-EGFR agents; it is probable therefore, that elderly NPC patients would be better candidates for such therapy.

Considering our cohort, these results suggest that EGFR expression in NPC is consistently associated with a high accumulation of p53 and Bcl-2. Remarkably, the absence of EGFR expression also correlated with a low p53 signal. We observed that the reverse situation was also true: a high p53 signal correlated with EGFR and Bcl-2 positivities, and a low p53 signal correlated with the absence of EGFR in all tumors tested. These observations are in line with previous reports suggesting an interaction between EGFR and p53 [26, 27] due to the presence of p53-responsive sites in the human EGFR gene promoter [28].

Additionally, we observed that up-regulation of Bcl-2 expression was more often associated with LMP1, EGFR and high p53 accumulation. When Bcl-2 was undetectable, the absence of LMP1 signal was common. Moreover, low levels of p53 and no EGFR were consistently detected. It is thus probable, that *in vivo*, Bcl-2 expression is linked, not only to LMP1 expression [11], but also to the EGFR/NF- κ B/Bcl-2 pathway [7, 8, 29-31].

Because, previous studies showed that up-regulation of EGFR or Bcl-2 in NPC is associated with poor prognosis and inhibition of chemotherapy-induced apoptosis [13, 32-34], it would be interesting to see if our observations could be linked to the fact that elderly patients with NPC are characterized by poor prognosis [35]. Whether any of the molecular signatures observed correlate with the high rates of cure observed in the juvenile form of the disease should also be addressed [36].

Collectively, our preliminary results indicate that Algerian and Tunisian patients with NPC share common features with regards to the expression of cellular and viral genes products. Our results require replication, but suggest a probable correlation between the accumulation of p53, Bcl-2 and EGFR. Moreover, they are indicative of a probable existence of a dominant molecular signature in elderly patients. To verify these points, it would be interesting to perform further studies using a larger number of patients. If replicated, these results could contribute to a better understanding of the oncogenic mechanisms of NPC in North Africa, and demonstrate the importance of establishing a molecular profile prior to considering EGFR-targeted therapy in NPC patients.

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