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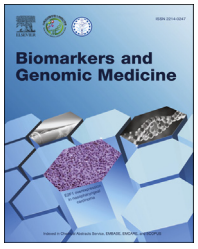

ORIGINAL ARTICLE

E2F transcription factor 1 overexpression as a poor prognostic factor in patients with nasopharyngeal carcinomas

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Abstract Nasopharyngeal carcinoma (NPC) is an endemic head and neck epithelial malignancy in Southeastern Asia and Taiwan. The E2 factor (E2F) family of transcription factors is downstream targets of the retinoblastoma protein 1. The E2F family of transcription factors

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survival

is the key regulator of genes involved in cell cycle progression, cell fate determination, DNA damage repair and apoptosis. E2F1 is unique in that it contributes both to the control of cellular proliferation and cellular death. However, the expression of E2F1 protein and its clinicopathological associations in patients with NPC are yet to be evaluated. Immunoexpression of E2F1 was retrospectively assessed in biopsies of 124 consecutive NPC patients without initial distant metastasis and treated with consistent guidelines. The outcomes were correlated with clinicopathological features and patient survivals. Results indicated that high E2F1 protein level (50%) was correlated with primary tumor ($p < 0.001$) and stage ($p = 0.002$; 7th American Joint Committee on Cancer). In multivariate analyses, high E2F1 expression emerged as an independent prognosticator for worse disease-specific survival ($p = 0.003$), distal metastasis-free survival ($p = 0.003$), and local recurrence-free survival ($p = 0.039$). In conclusion, high E2F1 protein level is common, associated with adverse prognosticators, and might confer tumor aggressiveness through tumor cell proliferation and metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic head and neck epithelial malignancy in Southeastern Asia and Taiwan; strongly linked to Epstein-Barr virus.[1,2] The latter association is especially authentic for the differentiated and undifferentiated nonkeratinizing carcinoma types, according to current World Health Organization (WHO) tumor classification, although genetic and environmental factors also play certain roles in pathogenesis.[1–3] The advances in diagnostic imaging, radiation therapy, and adjuvant chemotherapy of NPC have achieved better locoregional control, whereas it appears less satisfactory in final treatment outcomes.[4,5] Despite being an important parameter, tumor size, nodal status, and metastasis (TNM) staging still has space to improve in terms of providing the optimal prognostication to the patients.[1,4–6] Therefore, to identify potential biomarkers with better correlation to tumor growth and/or treatment outcomes in patients with NPC, subsequently, to aid in risk stratification and perhaps development of therapeutic targets, is indispensable.

The importance of the E2F/retinoblastoma 1 (RB1) pathway is underlined by its highly evolutionary conservation across the human,[7] and several model organisms.[8–10] In mammals, E2F activity is generated by a large number of interconnected complexes—E2Fs (E2F1-8),[7,11] transcription factor Dp family (TFDP1-4)[12,13] and three pocket proteins [RB1, retinoblastoma-like 1 (RBL1, also known as p107), RBL2 (also known as p130)].[14] Among E2Fs, E2F1, -2, and -3a are activators,[12,15] whereas E2F3b, E2F4, -5 and -6 are repressors via recruitment of transcriptional inhibitors such as histone deacetylase 1 or other chromatin remodeling factors to E2F-responsive promoters and resulting in transcriptional repression[16,17]; E2F7 and E2F8, lacking of sequences required for retinoblastoma 1 (RB1) family protein binding, are E2F1 inhibitors.[18] Physically, RB1, RBL1, and RBL2 interact with E2F1—6 transcription factors appear to be central to their roles in governing DNA replication.[7,19–21]

The role of E2F1 protein as an oncogene or tumor suppressor is still in debate at the present time.[22] The gene encodes for E2F1 was mapped to 20q11, one chromosomal region, which is frequently amplified in NPC-derived cell lines[23,24] and NPC tissue specimens,[25,26] suggesting that E2F1 might play an oncogenic role in patients with NPC. However, the expression profile of E2F1 in patients with NPC has not been evaluated. We therefore, aimed to systematically analyze E2F1 immunoexpression and its associations with clinicopathological factors and patient survivals.

Materials and methods

Patients and tumor specimens

The institutional review board had approved the study by using formalin-fixed tissue of NPC for this study (IRB201303-001). Available paraffin-embedded tissue blocks were retrieved from 124 NPC patients who underwent biopsy between January 1993 and December 2002. These patients were free of distant metastasis at initial presentation. The histological subtypes were reappraised according to the current WHO classification, and the tumor staging was re-evaluated with the 7th American Joint Committee on Cancer (AJCC) system by two pathologists independently.

Immunohistochemical staining and assessment of E2F1 expression

Tissue sections of 3-μm thickness were cut onto precoated slides from paraffin-embedded tissue blocks and were next routinely deparaffinized with xylene and rehydrated with ethanol washes. Slides were heated by the microwave in a 10 mM citrate buffer (pH 6.0) for 7 minutes to retrieve antigens. Endogenous peroxidase was blocked with 3% H_2O_2. Slides were next washed by Tris-buffered saline for 15 minutes and subsequently incubated with a primary polyclonal antibody targeting E2F1 (No. sc-251, KH95, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:100 for 1 hour. Primary antibodies were detected using the DAKO ChemMate EnVision Kit (K5001, Carpinteria, CA, USA). The slides were incubated and developed with the secondary antibody for 30 minutes, and 3,3-diaminobenzidine for 5 minutes, followed by counterstained using Gill's Hematoxylin. The immunoexpression of

E2F1 was scored by two pathologists (CF Li and HY Huang) using a multiheaded microscope to reach a consensus for each case without prior knowledge of clinical and follow-up information. Scoring of E2F1 immunoreactivity was evaluated on the basis of a combination of both the percentage and intensity of positively stained tumoral nuclei to generate H-score, which was calculated using the following equation: H-score $= \Sigma P_i(i+1)$, where i is the intensity of stained tumor cells (0 to 4+) and P_i is the percentage of stained tumor cells for each intensity varying from 0% to 100%. Tumor with H-score $>$ median of all cases was regarded as E2F1 high expression.

Treatment and follow-up

All 124 patients with follow-up for outcome received a complete course of radiotherapy [RT, total dose $\geq$ 7,000 cGy, concomitant boost RT (1,800 cGy/day, weeks 1 through 6; 1,600 cGy second daily fraction, weeks 5 through 6] using Varian 6/100 (DOTmed, Inc., New York, NY, USA) and Clinac 1800 linear accelerator (MedWoW Ltd., Nicosia, Cyprus), and also cisplatin-based chemotherapy (two cycles of concurrent cisplatin 100 mg/m^2 Day 1 and Day 22) in the case of Stages II-IV diseases, based on the previously published protocol.[27] The method of RT was in general uniform within this period. All patients were regularly monitored after RT until death or their last appointment with the mean follow-up duration being 59.6 months (range: 4-117 months).

Statistical analysis

Statistics were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Chi-square test was used to compare the E2F1 expression status and various clinicopathological parameters. The endpoints analyzed were disease-specific survival (DSS), distal metastasis-free survival (DMeFS) and local recurrence-free survival (LRFS), calculated from the starting date of RT to the date of event developed. Patients lost to follow-up were censored on the latest follow-up date. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was performed to evaluate prognostic differences between groups. Multivariate analysis was carried out by the Cox proportional hazards model. However, as a component factor of the AJCC staging system, primary tumor (T) and nodal status (N) were not introduced in multivariate comparisons. For all analyses, two-sided tests of significance were used with $p < 0.05$ considered significant.

Results

Immunohistochemical expression of E2F1 and associations with clinicopathological variables in NPC specimens

As shown in Table 1, 124 cases of NPC consists of five keratinizing squamous cell carcinomas, 42 nonkeratinizing differentiated carcinomas, and 77 nonkeratinizing, undifferentiated carcinomas. A total of 95 males and 29 females with a mean age of 48.6 years (range, 20-83 years) were included. Seven cases were classified as Stage I, 31 cases as Stage II, 46 cases as Stage III, and 40 cases as Stage IV. The E2F1 immunoexpression was observed and successfully scored in all cases ($n = 124$) with a wide range of positively stained tumoral nuclei, varying from 5% to 95% (median, 50%). Compared to representative normal tissue (Fig. 1A) and squamous metaplasia (Fig. 1B), a total of 62 cases with H $<$ median were therefore classified as low-expressing group (Fig. 1C); another 62 cases showed H $\geq$ median (Fig. 1D). High expression of E2F1 was significantly associated with NPC cases featuring increment of T3/T4 ($p < 0.001$) and advanced Stage III/IV ($p = 0.002$; Table 2). However, no association between the E2F1 expression score and the remaining clinicopathological factors was found.

Table 1 Clinicopathologic features of 124 nasopharyngeal carcinomas.

Parameters	Category	*n*	%
Gender			
	Male	29	23.4
	Female	95	76.6
Age (y)			
	$\leq$50	80	64.5
	51-60	21	16.9
	61-70	15	12.1
	71-80	7	5.6
	81-90	1	1.0
Primary tumor (T)			
	T1	30	24.2
	T2	50	40.3
	T3	21	16.9
	T4	23	18.5
Nodal status (N)			
	N0	24	19.4
	N1	32	25.8
	N2	48	38.7
	N3	20	16.1
Stage			
	I	7	5.6
	II	31	25.0
	III	46	37.1
	IV	40	32.3
Histological grade			
	Keratinizing	5	4.0
	Nonkeratinizing	42	33.9
	Undifferentiated	77	62.1

Prognostic impacts of E2F1 expression in NPC

Patients with NPC more frequently progressed to disease-specific mortality with advanced T3/T4 ($p = 0.0289$), N2/N3 ($p = 0.0008$) and Stages III/IV ($p = 0.0020$). Whereas the development of DMeFS and LRFS were significantly associated with T3/T4 ($p = 0.0085$ and $p = 0.0180$, respectively), N2/N3 ($p = 0.0132$ and $p = 0.0160$, respectively), and Stages III/IV ($p = 0.0072$ and $p = 0.0026$, respectively) with a medium duration of 21 months for both DMeFS and LRFS. Of note, E2F1 high expression correlated to a more aggressive clinical course with a shorter DSS ($p = 0.0001$), DMeFS ($p = 0.0001$), and LRFS ($p = 0.0041$), respectively (Table 3; Fig. 2). In

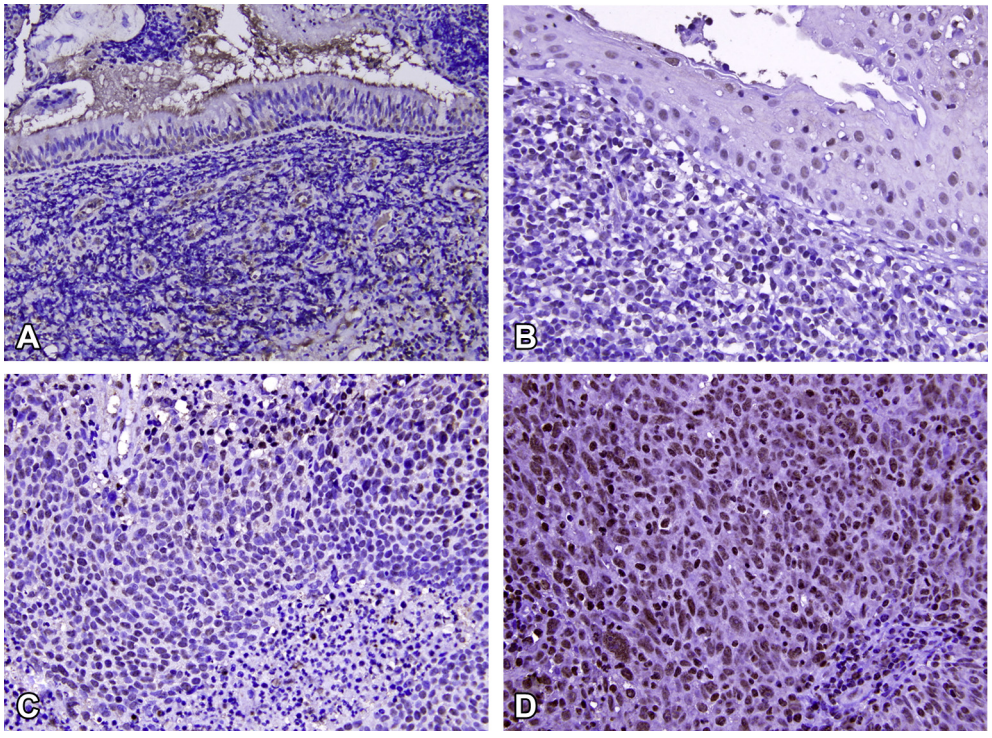

Figure 1 Immunoexpression of E2F1 is low in representative normal (A), squamous metaplasia (B), and (C) low-stage nasopharyngeal carcinoma (NPC); however, high in advanced stage (D) of NPC.

multivariate analysis, following tumor stage [$p = 0.027$, hazard ratio (HR) = 2.202 for DSS; $p = 0.019$; HR = 3.166 for LRFS], high E2F1 expression remained steady as a robust prognosticator for three endpoints evaluated and, independent portended inferior DSS ($p = 0.003$, HR = 2.353), DMeFS ($p = 0.003$; HR = 2.624), and LRFS ($p = 0.039$; HR = 2.072; Table 4). The survival months (mean ± SD) of DSS, DMeFS, and LRFS are also listed in Table 4.

Table 2 Associations between E2F1 expression and other important clinicopathologic variables.

Parameters	Category	E2F1 expression level		*p*
		Low	High	
Gender				
	Male	48	47	0.832
	Female	14	15	
Age (y)				
	<60	52	46	0.186
	≥ 60 years	10	16	
Primary tumor (T)				
	T1-T2	51	29	<0.001*
	T3-T4	11	33	
Nodal status (N)				
	N0-N1	33	23	0.071
	N2-N3	29	39	
Stage				
	I-II	27	11	0.002*
	III-IV	35	51	
Histological grade				
	Keratinizing	1	4	0.161
	Nonkeratinizing	25	17	
	Undifferentiated	36	41	

* Statistically significant.

Discussion

In this study, we demonstrate that high E2F1 protein level can be one potent prognosticator for DSS, DMeFS, and LRFS in patients with NPC, comparable with what were observed in other epithelial tumors.[28–31] For example, in a panel of 87 patients with nonsmall cell lung carcinoma (NSCLC), increased E2F1 proteins were dramatically associated with adverse prognosis.[28] In another cohort with 127 NSCLCs, quantitative RT-PCR identified that the overall survival rate was significantly lower in patients with high-*E2F1* tumors than in those with low-*E2F1* tumors.[29] Patients with higher E2F1 protein levels showed a notably shorter disease-associated survival time in R0 resection cases in pancreatic ductal carcinomas.[30] Besides, high E2F1 expression in patients with breast cancer displayed significantly worse survival.[31] Indeed, NPC might differ from other epithelial cancers in its etiology, epidemiology, and potential therapeutic options; despite having a cure for the majority of the patients, challenges still exist in the prevention of recurrence and treatment of patients with refractory or metastatic NPC.[32] Therefore, identifying biomarkers indepen dently correlated with tumor aggressiveness to facilitate appropriate allocation of adjuvant therapy will be valuable for patient-tailored strategies to manage high-risk NPCs.[33] We accordingly speculate that E2F1 might not only be a

Table 3 Univariate log-rank analyses.

Parameters	Category	*n*	DSS		DMeFS		LRFS	
			n	*p*	*n*	*p*	*n*	*p*
Gender								
	Male	95	45	0.7870	38	0.6128	30	0.3240
	Female	29	14		11		7	
Age (years)								
	<60	98	48	0.8600	42	0.3091	29	0.8206
	≥60	26	11		7		8	
Primary tumor (T)								
	T1-T2	80	32	0.0289*	25	0.0085*	19	0.0180*
	T3-T4	44	27		24		18	
Nodal status (N)								
	N0-N1	56	18	0.0008*	17	0.0132*	12	0.0160*
	N2-N3	68	41		32		25	
Stage								
	I-II	38	10	0.0020*	9	0.0072*	5	0.0026*
	III-IV	86	49		40		32	
Histological grade								
	Keratinizing/non-keratinizing	47	20	0.1980	17	0.2753	15	0.9521
	Undifferentiated	77	39		32		22	
E2F1 expression	Low (H-score < median)	62	19	0.0001*	15	0.0001*	13	0.0041*
	High (H-score ≥ median)	62	40		34		24	

DMeFS = distal metastasis-free survival; DSS = disease-specific survival; LRFS = local recurrence-free survival.
* Statistically significant.

potential therapeutic target but also a remedial marker in patient with NPC.

Significantly increased hazard ratios of DSS, DMeFS, and LRFS in NPC patients with higher stages, III-IV, were further ascertained, analogous to other studies.[34,35] In conjunction with meaningful correlations between the E2F1 protein level and both primary tumor and stage, we therefore suggest that high E2F1 protein might contribute to the tumor cell proliferation and metastasis in at least a subset of patients with NPC. Consistent with the results in this NPC cohort, upregulation of E2F1 protein has been identified in several types of tumor and is correlated with tumor cell proliferation, such as lung cancers,[28,29,36] osteosarcoma,[37] and squamous cell carcinoma of the oesophagus.[38,39] In these cases, E2F1 protein overexpression in neuroendocrine lung tumors was drastically associated with a high Ki-67 proliferative index and the B-cell CLL, lymphoma 2/BCL2-associated X protein ratio (BCL2/BAX > 1).[36] Because BLC2 and BAX are anti- and proapoptotic regulators, respectively, E2F1 apparently plays an oncogenic role. Likewise, high E2F1 immunostainings demonstrated a radical increase in their indexes of the proliferating cell nuclear antigen (PCNA) in NSCLCs.[28] Significant correlation has also been detected between the mRNA levels of *E2F1* and thymidylate synthase (*TYMS*)[29]; the latter encodes the enzyme used to generate thymidine monophosphate and is subsequently phosphorylated to thymidine triphosphate for use in DNA synthesis and repair.[40,41] In these NPC patients, we lately uncovered that high TYMS protein levels can serve as an independent prognosticator for inferior DSS, DMeFS, and LRFS, strengthens the underlying molecular relationship between E2F1 and TYMS in patients with NPC. In cellular and/or animal models, stable transfection of *E2F1* gene into low invasive, head and neck carcinoma-derived cell lines with low expression of *E2F1* dramatically induced their invasive ability.[42] Moreover, primary rat embryo cells that were transfected with *E2F1* and *TFDP1* form colonies in soft agar and induce tumor formation in nude mice.[43] Overexpression of *E2F1* in rat embryonic fibroblasts leads to neoplastic transformation.[44] Lentivirus-mediated RNA interference targeting *E2F1* inhibits human gastric cancer MGC-803 cell growth through upregulation of phosphatase and tensin homolog (also known as PTEN), caspase-3 and -9 expression levels and downregulation of nuclear factor kappa B in a mice xenograft model.[45] Mice implanted with metastatic SK-Mel-147 melanoma cells expressing *E2F1* short hairpin RNA had a significantly smaller area of metastases per lung than controls.[46] Therefore, findings from clinical associations, cellular, and animal models support our results.

Nevertheless, overexpression of *E2F1* was also substantially associated with increased disease-free survival in squamous cell carcinoma of the anterior tongue.[47] In human colorectal adenocarcinomas, the relation between E2F1 expression level and apoptosis was drastically correlated.[48] Nuclear E2F1 expression was significantly and inversely correlated with phospho-RB1 and positively related to tumor apoptotic index in patients with hepatocellular carcinoma.[49] Low *E2F1* transcript levels are a strong determinant of favorable breast cancer outcome, with low risk of metastasis irrespective of estrogen receptor status.[50] Furthermore, E2F1 overexpression correlates with decreased proliferation and better prognosis in adenocarcinomas of Barrett esophagus.[51] In most cellular models, high E2F1 levels exerted the growth-suppressing or proapoptotic effect, including Yes-4 and Yes-6 cells of esophageal cancer,[52] MKN-45 cells of gastric carcinoma,[53] and osteosarcoma-derived Saos2 cells.[54]

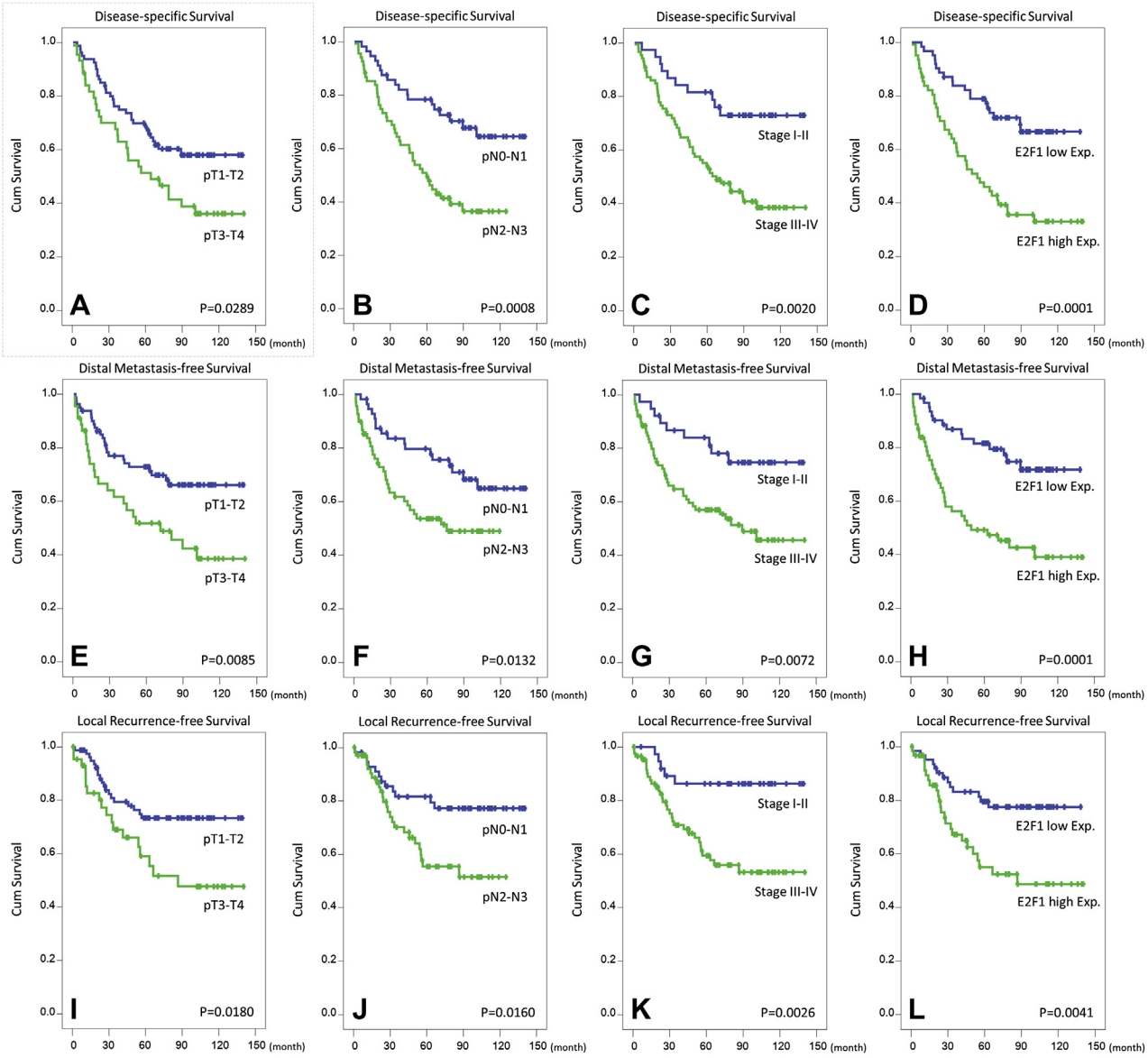

Figure 2 Kaplan-Meier plots show that advanced primary tumor (T3-T4; A, E, I), nodal status (N2-N3; B, F, J), and stage (III-IV; C, G, K), as well as E2F1 high expression (D, H, L) impact significantly inferior prognostic outcomes in disease-specific survival (A-D), distal metastasis-free survival (E-H), and local recurrence-free survival (I-L).

However, these observations are not completely controversial because *E2F1* was recognized as a sturdy regulator of apoptosis upon DNA damage in all human cancer types originally.[55] Hypophosphorylated (active) RB1 binds E2F1 and thereby downregulates E2F1 activity, suggesting a model in which RB1 restricts cell cycle progression by restraining E2F1.[7,14,56]

It has been shown that tumor cells, especially from advanced lesions, exhibit severe defects in the cell death pathways that are normally activated by E2F1, which may otherwise select against apoptotic consequences of deregulated E2F1 in the absence of RB1.[57] Whether the balance of E2F1 activity in a specific tissue inhibits or promotes tumorigenesis is most likely dependent upon the background of pro versus anti-aporpotic signals received by cells at a given time.[58] Transactivation of E2F1 target genes, *TYMS*, dihydrofolate reductase, *PCNA*, ribonucleotide reductase M1, cyclin E1, cyclindependent kinase 1, myeloblastosis viral oncogene homolog (avian)-like 2,[59,60] and stathmin 1,[61] that participate in the processes of angiogenesis, invasion, and metastasis, along with significantly higher E2F1 protein levels were expressed in advanced lesions with large tumor size (T3-T4) and progressive stage (Stages III-IV) in this NPC cohort, reinforce the view that E2F1 plays a central role in many aspects of NPC development.[62]

Taken together, prognostic evaluation of E2F1 protein level in a well-characterized, large series of NPC were performed. The E2F1 was detectable by immunohistochemistry in the vast majority of NPC but shows a wide range of distribution in expression level, as assessed by H-score. Along with higher tumor stage, E2F1 overexpression is independently predictive of DSS, DMeFS, and LRFS, additionally, which might represent a useful prognostic adjunct to better stratify the prognosis of NPC cases.

Table 4 Multivariate survival analyses.

Parameter	Category		DSS			DMeFS			LRFS		
		Survival months (mean ± SD)	(95% CI) HR	*p*	Survival months (mean ± SD)	(95% CI) HR	*p*	Survival months (mean ± SD)	(95% CI) HR	*p*
Stage			(1.095-4.427)	0.027*		(0.937-4.159)	0.074		(1.208-8.295)	0.019*
	I-II	82.4 ± 34.8	1		80.7 ± 36.6	1		80.2 ± 37.1	1	
	III-IV	60.2 ± 37.7	2.202		54.9 ± 39.4	1.974		53.0 ± 38.8	3.166	
E2F1 Expression			(1.343-4.124)	0.003*		(1.403-4.907)	0.003*		(1.038-4.138)	0.039*
	Low (H-score < median)	76.3 ± 32.6	1		73.3 ± 34.0	1		72.3 ± 35.8	1	
	High (H-score ≥ median)	57.7 ± 41.1	2.353		52.4 ± 43.4	2.624		50.3 ± 41.5	2.072	

CI = confidence interval; DMeFS = distal metastasis-free survival; DSS = disease-free survival; HR = hazard ratio; LRFS = local recurrence-free survival; SD = standard deviation.
* Statistically significant.

Acknowledgments

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