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Original Paper

Distinct expression patterns of the transcription factor E2F-1 in relation to tumour growth parameters in common human carcinomas

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Abstract

E2F-1 is a pivotal transcription factor that integrates signals from a variety of G1/S phase regulators and modulates diverse cellular functions, such as DNA synthesis, repair, mitosis, and apoptosis. Its role in cellular proliferation and apoptosis, as depicted from experimental models and limited reports in human malignancies, remains a matter of debate. Recently, in non-small cell lung cancer, it was observed that E2F-1 overexpression was associated with tumour growth, implying an 'oncogenic' effect. To clarify further the role of E2F-1 in carcinogenesis, the investigation was expanded in four of the most common human malignancies by examining its expression status and putative impact on tumour kinetics. These issues were addressed by immunohistochemical and molecular means in 52 breast carcinomas, 42 prostate adenocarcinomas, 58 colon adenocarcinomas, and 77 superficial bladder transitional cell carcinomas (TCCs). The following results were found: (i) in breast carcinomas, E2F-1 expression correlated with proliferation ($p < 0.001$) and growth index ($p = 0.001$); (ii) in prostate adenocarcinomas, absence of E2F-1 was noted, in contrast to its expression in normal and hyperplastic glands; (iii) in colon adenocarcinomas, E2F-1 expression was inversely related to growth index ($p = 0.001$), being expressed in lesions with increased apoptosis ($p = 0.001$) and low proliferation ($p < 0.001$); and (iv) in superficial TCCs, E2F-1 expression correlated with proliferation ($p = 0.002$). Taken together, these results suggest that E2F-1 has a growth-promoting effect in breast carcinomas and superficial TCC, whereas the opposite seems to be the case for colon and prostate cancer. To interpret the above findings, the status of the pRb and p53 tumour suppressor pathways, which are known to affect E2F-1 activity, was further investigated. The results suggest that the actions of E2F-1 are mainly dependent on the functionality of these pathways. Nevertheless, the data also imply that p53-independent pathways may play a nodal role in the function of E2F-1 in colon cancer.

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Introduction

Pathways governing the transition from the G1 to the S phase of the cell cycle are frequent targets during carcinogenesis, since they allow the cancer cell to acquire autonomy from regulatory signals of the microenvironment.

The E2F-1 transcription factor plays a key role in G1 to S-phase transition by attracting numerous upstream signals, determining whether the cell should advance through the cell cycle or die via apoptosis [1,2]. It belongs to the subgroup of activators (E2F-1, 2, and 3) of the E2F transcription factor family [2]. E2F-1 induces the expression of a wide spectrum of genes implicated in DNA synthesis, repair, cell-cycle control, and apoptosis [3–5]. The transcriptional activity of E2F-1 is negatively regulated by the pRb 'pocket' protein, which 'masks' its transactivation domain. Additionally, histone deacetylase 1 (HDAC1), which suppresses transcription, is recruited to modify the histones of the target promoter regions. Cyclin–cdk-dependent phosphorylation of pRb removes this inhibition, releasing E2F-1 transcriptional activity [6,7].

Initially, E2F-1 was suggested to behave as an oncogene; however, recent studies with knockout mice have shown that it may also function as a tumour suppressor gene (TSG). E2F-$1^{(-/-)}$ mice demonstrated a bimodal phenotype facilitating tumour development in some tissues, while inhibiting tumour formation in others [8–10]. The basis of this behaviour is undetermined and it is unclear whether it also applies in human tumours.

Reports regarding the relationship between E2F-1 and tumour kinetics are limited in lung cancer [11,12], whereas in certain other malignancies, the data are incomplete [13–17]. Recently we observed, in non-small cell lung carcinomas (NSCLCs), that E2F-1 expression correlated with the growth indices of the carcinomas, within the context of aberrant pRb and p53 status [11].

To clarify the role of E2F-1 in carcinogenesis further, we expanded our investigation using breast carcinomas (BrCs), prostate adenocarcinomas (PrCs), colon adenocarcinomas (CCs), and transitional cell carcinomas of the bladder (TCCs), four of the most common human malignancies [18]. We examined its expression status and relationship to tumour kinetic parameters. Furthermore, the results were correlated with the status of the pRb and p53 pathways, which have been shown to affect E2F-1 activity directly and indirectly [2]. To the best of our knowledge, such a comprehensive and comparative '*in vivo*' study dealing with E2F-1 has not been reported to date.

Table 1. Summary of the clinicopathological features and kinetic parameters in the carcinomas examined

Breast carcinomas (52 cases)*
Histology: DCs 47, LCs 2, MICs 3
Age: Years 55–77
Stage (19): I 8, II 25, III 15, IV 4
Grade: I 5, II 31, III 16
Estrogen acceptors: Positive 40, negative 12
Progesteron receptors: Positive 33, negative 19
Survival status (follow-up 61 months): Deaths 16, alive 36
Proliferation index: M 18.9%, SD 9.8%, range 5–42%
Apoptotic index: M 1.66%, SD 0.38%, range 1.0–2.3%
Growth index: M 11.3%, SD 5.8%, range 2–26%
Prostate adenocarcinomas (42 cases)*
Age: Years 55–78
Gleason score (20): 2–4 12, 5–6 12, 7 6, 8–10 12
Colon adenocarcinomas (58 cases)*
Age: Years 57–78
Stage: A 5, B 34, C 16, D 3
Survival status (follow-up 59 months): Deaths 8, alive 50
Proliferation index: MD 40.00%, range 5–90%
Apoptotic index: MD 1.66%, range 0.5–3.5%
Growth index: M 38.3%, SD 22.6%, range 2–81%
Superficial transitional cell carcinomas (77 cases)*
Age: Years 41–93
Smoking: Positive 59, negative 18
Stage (22): Ta 50, T 27
Grade: I 12, II 63, III 2
Survival status (follow-up 47 months): Recurrence 27
Proliferation index: MD 15.0%, range 5–45%
Apoptotic index: MD 1.00%, range 0.5–3.5%
Growth index: MD 17.5%, range 3–70%

* Material comprised formalin-fixed, paraffin wax-embedded tissues. Also, for 15 BrCs and 20 CCs, frozen material from matched normal/tumour samples was available. DC-ductal carcinoma; LC-lobular carcinoma; MIC-mixed invasive carcinoma; M-mean value; SD-standard deviation, for normal distribution; MD-median value, for non-normal distribution.

Materials and methods

Tissue samples

The collection, coding, and analysis of the tissues and data banks were performed in accordance with the revised (1983) Helsinki Declaration of 1975 for ethical standards, as well as governmental guidelines issued by the Greek National Committee for the protection of personal information disclosure. All cases were collected from the Department of Pathology. The patients had not undergone any therapy prior to surgery and had no history of familial cancer. The clinicopathological data of all the cases are presented in Table 1.

Cell cultures

The MCF-7 breast and PC-3 prostate carcinoma cell lines (ATCC, Manassas, VA, USA) were cultured according to the supplier's instructions.

Immunohistochemistry

Antibodies (Abs)

The antibodies used were as follows: from Santa Cruz, Bioanalytica, Greece: mouse anti-E2F-1 (KH95), rabbit anti-E2F-2 (L-20), mouse anti-pRb (IF-8), goat anti-phospho-pRb (p-Rb Ser-795), and mouse anti-$p16^{INK4A}$ (F-12); from Dako, Kalifronas, Greece: mouse anti-Ki-67 (MIB-1), mouse anti-p53 (DO7), and mouse anti-$p27^{Kip1}$ (SX53G8).

Method

Immunohistochemistry was performed as previously described [11].

Evaluation and controls

Evaluation of E2F-1 and proliferation indices (PIs), as well as assessment of p53, pRb, $p16^{INK4a}$, and $p27^{Kip1}$ immunostaining, was carried out according to criteria described elsewhere [11,23–25].

Indirect immunofluorescence

The anti-E2F-1 and anti-E2F-2 (1 : 100 dilutions), FITC-labelled goat anti-mouse and RISH-labelled goat anti-rabbit complexes (1 : 250 dilutions) (Santa Cruz, Bioanalytica, Greece) were used as primary and secondary antibodies, respectively. Counterstaining was performed with DAPI (Sigma, Greece).

5. Tdt-mediated dUTP nick end labelling assay (TUNEL)

The method applied, as well as the estimation of the apoptotic index (AI), has been previously described [25].

Comparative reverse transcription (RT)-PCR

RNA extraction and cDNA synthesis were performed on available frozen samples, as previously described [11]. *E2F-1* mRNA levels were assessed with a semi-quantitative multiplex RT-PCR method, as described elsewhere [11].

Protein extraction and western blot analysis

Protein extraction from available frozen samples and western blot analysis for E2F-1 were performed as previously described [11].

DNA extraction, mutation, and allelic imbalance (AIm/LOH) analysis

*p*53 was screened by nested PCR/SSCP on matched normal/tumour DNA and mutations were sequenced automatically, as previously described [23]. On the same samples, AIm analysis for *p*53 was performed using the internal pentanucleotide marker *D17S179E* [25].

Statistical analysis

Associations of E2F-1 expression with clinicopathological parameters, and the status of pRb and p53, were assessed using the *t*-test or analysis of variances, as appropriate. The relationship of E2F-1 with the proliferation index (PI), apoptotic index (AI), and growth index (GI) (= PI/AI) was examined with bivariate correlations (Pearson's or Spearman's test). Overall and recurrence-free survival analysis was performed by Kaplan–Meier methodology and Cox regression analysis. All tests were performed with SPSS-10 (SPSS Inc, Chicago, IL, USA). Statistical associations were considered significant for p values less than 0.05.

Results

E2F-1 status and relationship with clinicopathological parameters

E2F-1 protein status was assessed as the percentage of nuclear stained tumour cells (E2F-1 index, EI).

Breast cancer (Figure 1A)

Significantly higher EI values (range 15–45%, mean value $29.5 \pm 7.8\%$) were observed in the tumour areas than in normal mammary glands ($6.2 \pm 2.3\%$) ($p < 0.001$). Immunohistochemical results were confirmed by comparative RT-PCR and western blot analysis of the available frozen material. A significant correlation between EI and stage of the disease was found (I/II: $27.9 \pm 5.6\%$ versus III: $30.5 \pm 6.6\%$ versus IV: $38.5 \pm 6.0\%$, $p = 0.005$).

Colon cancer (Figure 1B)

E2F-1 staining was seen in normal and cancerous tissue. In normal mucosa, its staining was restricted to the base of the colonic glands. In the cancerous areas, EI ranged from 5% to 80%, with a mean value of 23.6 ± 17.5. Immunohistochemical results were confirmed by comparative RT-PCR and western blot analysis in all cases with available frozen material.

Prostate cancer (Figure 1C)

E2F-1 immunoreactivity was absent in the tumour areas in contrast to the strong nuclear signal identified in the basal cells of normal and hyperplastic prostate glands (Figure 1C, i–iii). Interestingly, a clear E2F-2 nuclear signal was seen in tumour cells as well as in the basal cells of the normal and hyperplastic prostate glands (Figure 1C, iv and v). Notably, in several cases, cytoplasmic staining was observed. Similarly, absence of E2F-1 immunofluorescence staining was observed in the PC-3 prostate cell line, whereas in the same cell line a clear nuclear signal for E2F-2 was observed (Figure 1C, vi).

Superficial TCC (Figure 1D)

E2F-1 immunoreactivity ranged from 24% to 53%, with a mean value of $37.5 \pm 8.5\%$. E2F-1 immunoexpression was higher in the tumour cells next to the stroma. In the normal transitional bladder epithelium, E2F-1 immunopositivity was restricted to the cells of the basal layer.

In colonic cancers and bladder TCCs, no association between EI and clinicopathological features was recorded. It is noteworthy that only scanty E2F-1-positive stromal cells were observed in all of the carcinomas examined.

Relationship of E2F-1 status with tumour kinetic parameters

A summary of the kinetic parameters (proliferation and apoptotic indices) of the BrCs, CCs and TCCs examined are presented in Table 1.

Breast cancer

Assessment of E2F-1, Ki-67, and apoptosis in serial sections showed co-localization of the former two in the tumour areas (Figure 2A). These *in situ* observations were further confirmed statistically, since EI was

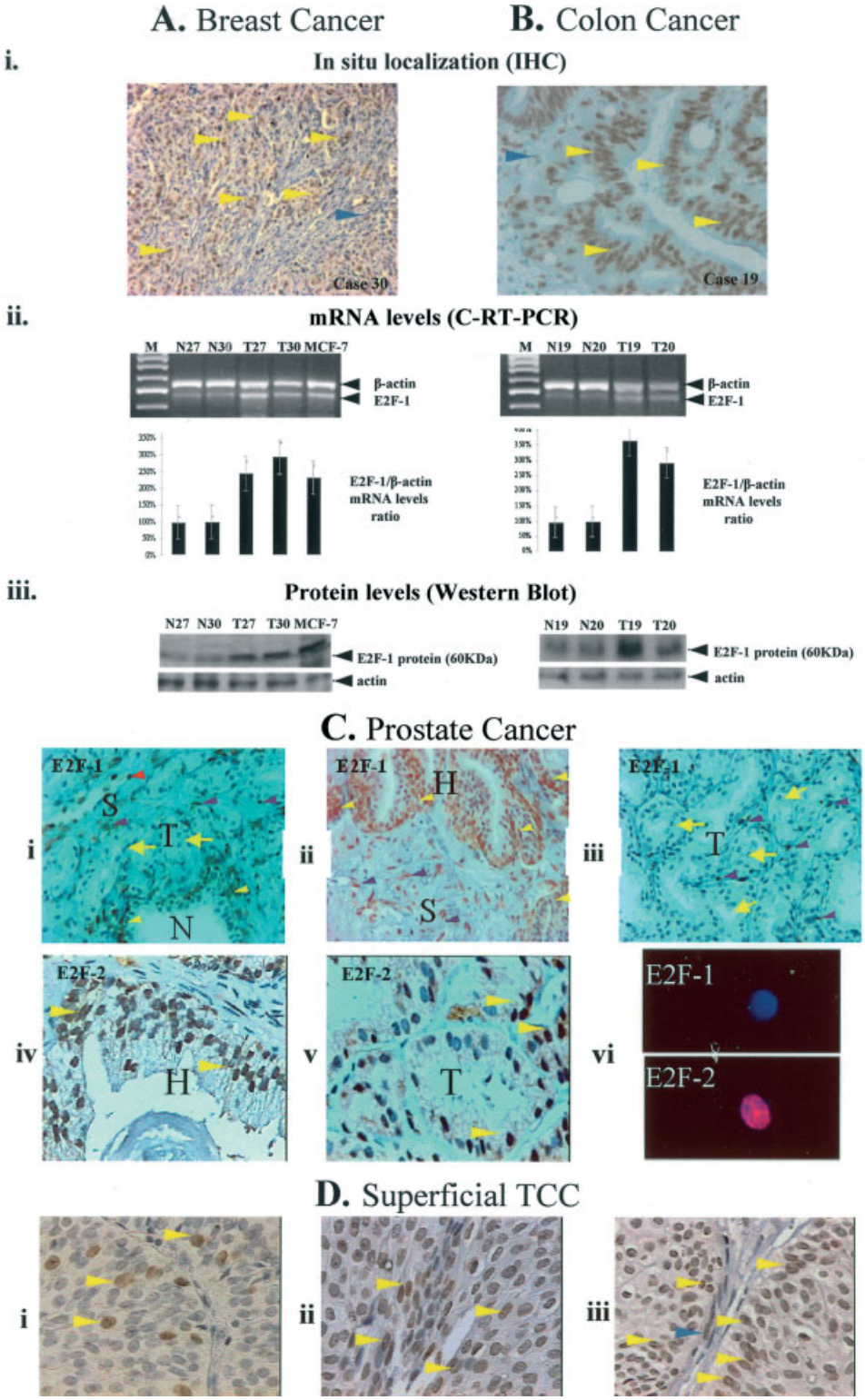
significantly associated with GI and PI but not AI (Figure 2B).

Colon cancer

Evaluation of E2F-1 and kinetic parameters in serial sections revealed that tumour areas with elevated E2F-1 expression were characterized by low proliferation and high apoptosis (Figure 3A). In lesions that exhibited decreased E2F-1 staining, the opposite was found. These results were also confirmed statistically (Figure 3B).

TCC

Examination of E2F-1 and kinetic determinants in serial sections showed co-localization of all three parameters in the tumour region (Figure 4A). Statistically, a positive correlation was observed only between increased E2F-1 immunoreactivity and PI (Figure 4B).



Furthermore, an association between PI and AI was observed (Spearman's test, $r = 0.322$, $p = 0.052$).

Survival analysis

Setting the E2F-1 median value (for BrCs: 30%, for CCs: 20%, and for TCCs: 35%) as a cut-off point and applying Kaplan–Meier methodology, we found a significant association between increased EI and poor patient outcome in BrCs ($p < 0.001$), favourable prognosis in CCs ($p = 0.029$), and post-annual disease recurrence in TCCs ($p = 0.020$). However, Cox regression analysis showed that E2F-1 expression, in

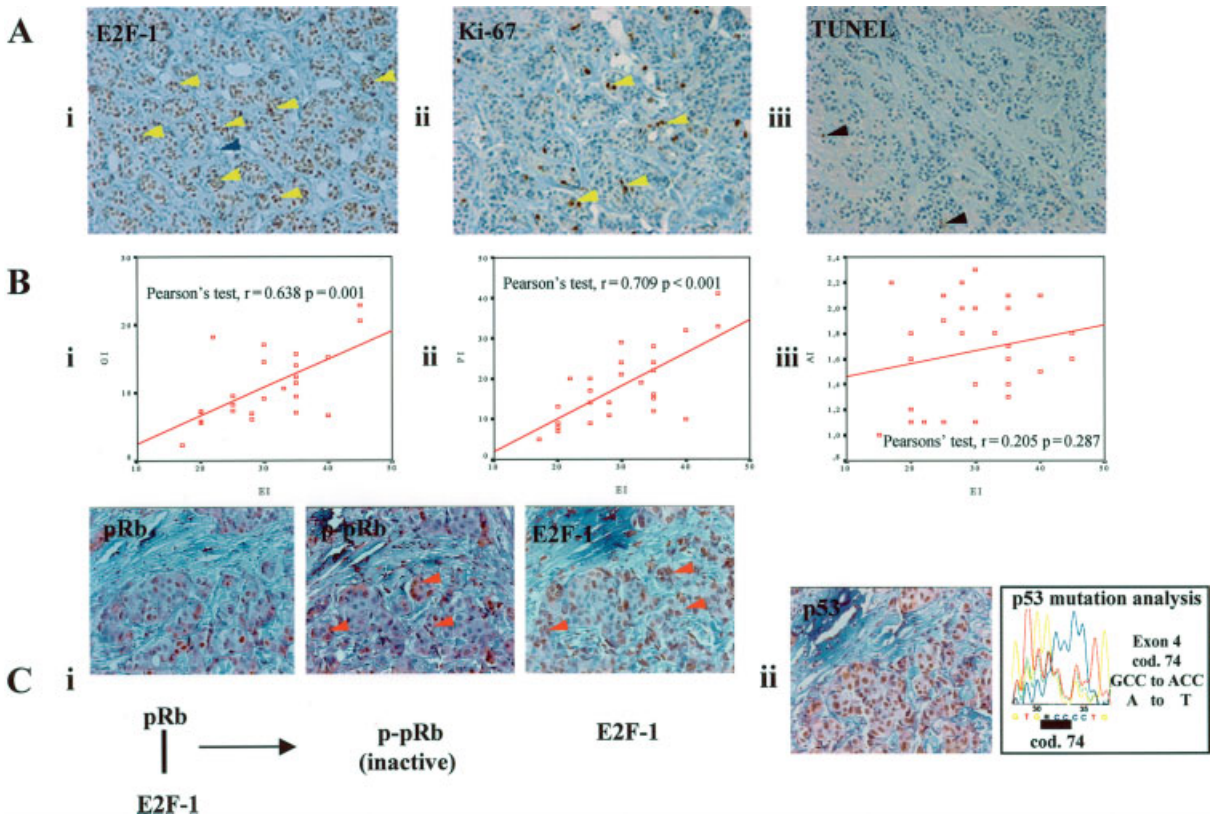


Figure 2. Relationship of E2F-1 status with tumour kinetics, pRb and p53 pathways in breast cancer. (A) Representative results of E2F-1 and Ki-67 immunohistochemistry and TUNEL assay in serial sections. Yellow arrow-heads depict E2F-1 and Ki-67 immunostaining in tumour cells; the blue arrow-head shows E2F-1 staining in stromal cells; the black arrow-head illustrates apoptotic nuclei stained by TUNEL assay. (B) Scatter plots and the corresponding fitted lines depicting the correlation between EI and GI (i), PI (ii), and AI (iii), respectively. The results of the appropriate bivariate statistical tests are also presented. (C) Representative results for pRb and p53 status. (i) pRb and phosphorylated pRb (inactive) as well as E2F-1 were evaluated by immunohistochemistry in serial sections. Red arrow-heads depict phosphorylated pRb and E2F-1 staining in the tumour area. (ii) Positive p53 immunostaining as a result of *TP53* mutation as verified by automated sequencing

Figure 1. E2F-1 analysis in common human carcinomas: (A) Breast Cancer. (i) Representative results of E2F-1 immunohistochemistry (case 30). Yellow arrow-heads depict E2F-1 staining in tumour cells; the blue arrow-head shows E2F-1 staining in stromal cells. (ii) Comparative (C-) RT-PCR demonstrating increased E2F-1 mRNA levels in BrC. M = 100 bp ladder (Fermentas, BioSure, Greece). Results of the RT-PCR analysis from two normal mammary samples (cases 27 and 30) (lanes N27 and N30), their corresponding tumours (lanes T27 and T30), and the MCF-7 breast cell line (lane MCF-7). (iii) Representative results from western blot analysis performed on the same cases mentioned above showing increased E2F-1 protein levels. (B) Colon cancer. (i) Representative results of E2F-1 immunohistochemistry. Yellow arrow-heads depict E2F-1 immunopositivity in tumour; the blue arrow-head shows E2F-1 staining in stromal cells. (ii) Comparative (C-) RT-PCR demonstrating increased E2F-1 mRNA levels in CC. M = 100 bp ladder (Fermentas, BioSure, Greece). Results from comparative RT-PCR analysis in two normal colon samples (cases 19 and 20) (lanes N19 and N20) and their corresponding tumours (lanes T19 and T20). (iii) Representative results of western blot analysis performed in the same cases mentioned above showing increased E2F-1 protein levels. (C) Prostate cancer. In panels i–iii, representative immunohistochemical results of E2F-1 staining in needle biopsies containing prostate carcinoma are shown. S = stroma; T = tumour; N = normal gland; H = hyperplastic gland. Yellow arrows show absence of E2F-1 immunoreactivity; yellow arrow-heads depict E2F-1 immunopositivity in normal and hyperplastic gland; blue arrow-heads show E2F-1 staining in stromal cells; the red arrow-head illustrates E2F-1 immunoreactivity in vascular endothelium. In panels iv and v, representative immunohistochemical results of E2F-2 staining in needle biopsies containing prostate carcinoma are shown. T = tumour; H = hyperplastic gland. Yellow arrow-heads depict E2F-2 immunopositivity in tumour and hyperplastic gland. Positivity of the PC-3 cell line for E2F-2, but not E2F-1, by immunofluorescence is shown in vi. (D) Superficial TCC. Representative results of three cases exhibiting low (i), medium (ii), and high E2F-1 immunoreactivity (iii). Yellow arrow-heads depict E2F-1 staining in tumour cells; the blue arrow-head shows E2F-1 staining in stromal cells

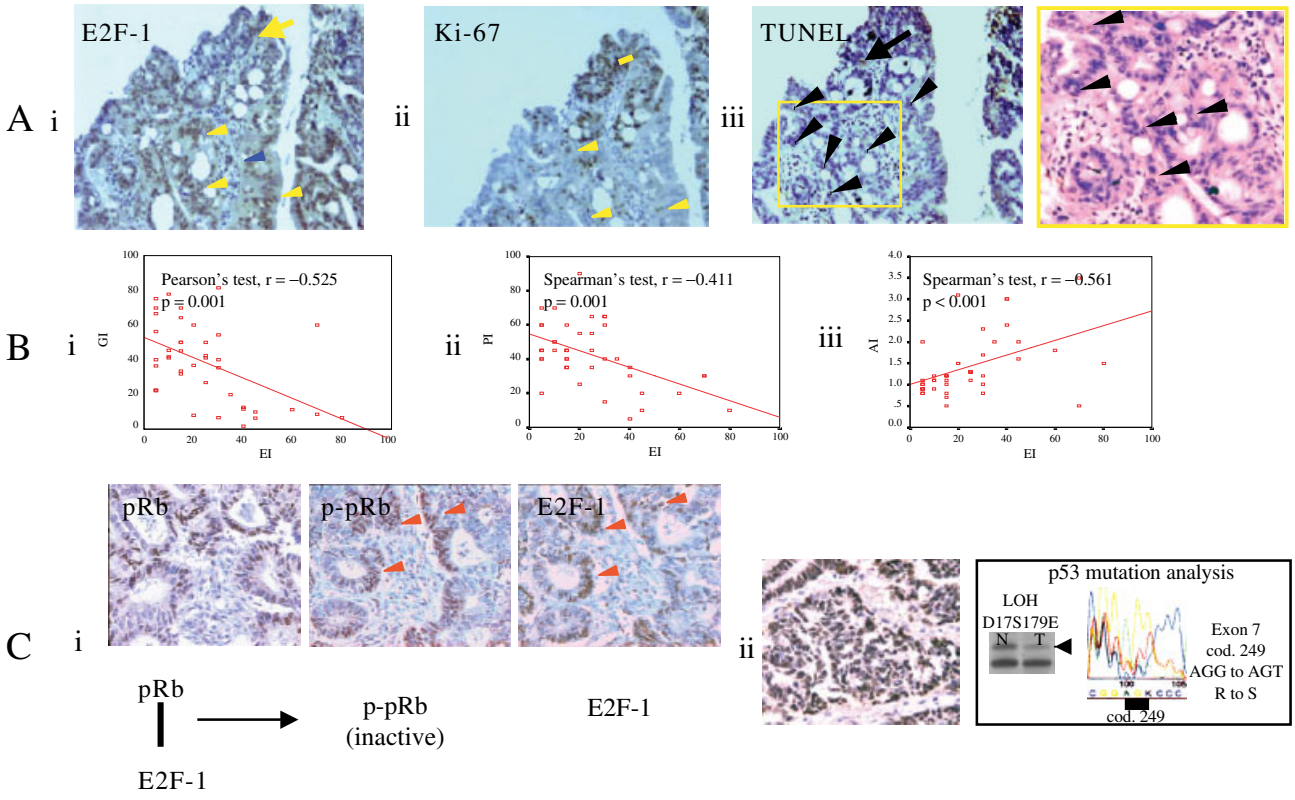


Figure 3. Relationship of E2F-1 status with tumour kinetics, pRb and p53 pathways in colon cancer. (A) Representative results of E2F-1 and Ki-67 immunohistochemistry and TUNEL assay in serial sections of colon cancer. Yellow arrows show tumour cells with no E2F-1 immunoreactivity but positive staining for Ki-67; yellow arrow-heads depict tumour areas with high E2F-1 immunopositivity and low Ki-67 labelling; the blue arrow-head shows E2F-1 staining in stromal cells; and the black arrow-head and arrow illustrate apoptotic nuclei stained by TUNEL assay in a region with presence and absence of E2F-1 immunoreactivity, respectively. The region included in the yellow rectangle in A, iii is shown in magnification in the right-hand panel. (B) Scatter plots and the corresponding fitted lines depicting the correlation between EI and GI (i), PI (ii), and AI (iii), respectively. The results of the appropriate bivariate statistical tests are also presented. (C) Representative results for pRb and p53 status. (i) pRb and phosphorylated pRb (inactive) as well as E2F-1 were evaluated by immunohistochemistry in serial sections. Red arrow-heads depict phosphorylated pRb and E2F-1 staining in tumour cells. (ii) Positive p53 immunoreactivity as a result of *TP53* aberrations (loss of one allele accompanied by mutation of the other allele) as verified by LOH and automated sequencing analysis

contrast to stage of disease, was not an independent survival factor in any of the carcinomas examined.

Relationship of E2F-1 status to the pRb and p53 cell-cycle regulatory pathways

The apparently contradictory expression patterns of E2F-1 in relation to growth prompted us to investigate, in our series of tumours, the status of the two major tumour suppressor pathways of the cell, pRb and p53, which directly and indirectly affect E2F-1 activity [2,4].

Breast cancer

We previously investigated alterations of pRb and cyclin-dependent kinase inhibitors (CKIs) $p16^{INK4a}$ and $p27^{Kip1}$ in the same set of breast tumours [24,26]. Considering the cumulative information for the status of these cell-cycle regulators, we found that tumours with abnormal expression of the $p16^{INK4a}/p27^{Kip1}$/pRb network were associated with increased E2F-1 expression (*t*-test, $p = 0.016$) (Figure 2C, i). Notably, we observed that phospho-pRb (p-pRb) immunoreactivity paralleled E2F-1 expression in the cases with intact pRb, suggesting that, in these samples, E2F-1 immunoreactivity predominantly represents the form of E2F-1 that is released from pRb. Subsequently, we noticed p53 immunopositivity in 14 cases (26.9%), which was concordant with the presence of mutations (10/14 –71.4%), but not with AIm (Table 2 and Figure 2C, ii). AIm analysis at the *p53* locus revealed loss in 20 breast carcinomas (38.5%). Thus, overall, p53 alterations were observed in 30/52 (57.7%) patients. Examining the status of E2F-1 in the patients with wild-type and defective p53, we observed a significant association between the latter and high E2F-1 levels ($31.1 \pm 6.3\%$ versus $27.4 \pm 6.3\%$, $p = 0.042$ by *t*-test).

Colon cancer

Investigating the relationship between E2F-1 and pRb, we observed that the latter was universally expressed in the tumour areas in the majority of cases. However, the inactivated form of pRb, p-pRb, co-localized strictly with E2F-1, indicating that the latter, in a significant portion, corresponds to the free and active form (Figure 3C, i). Examining the status of p53 in our

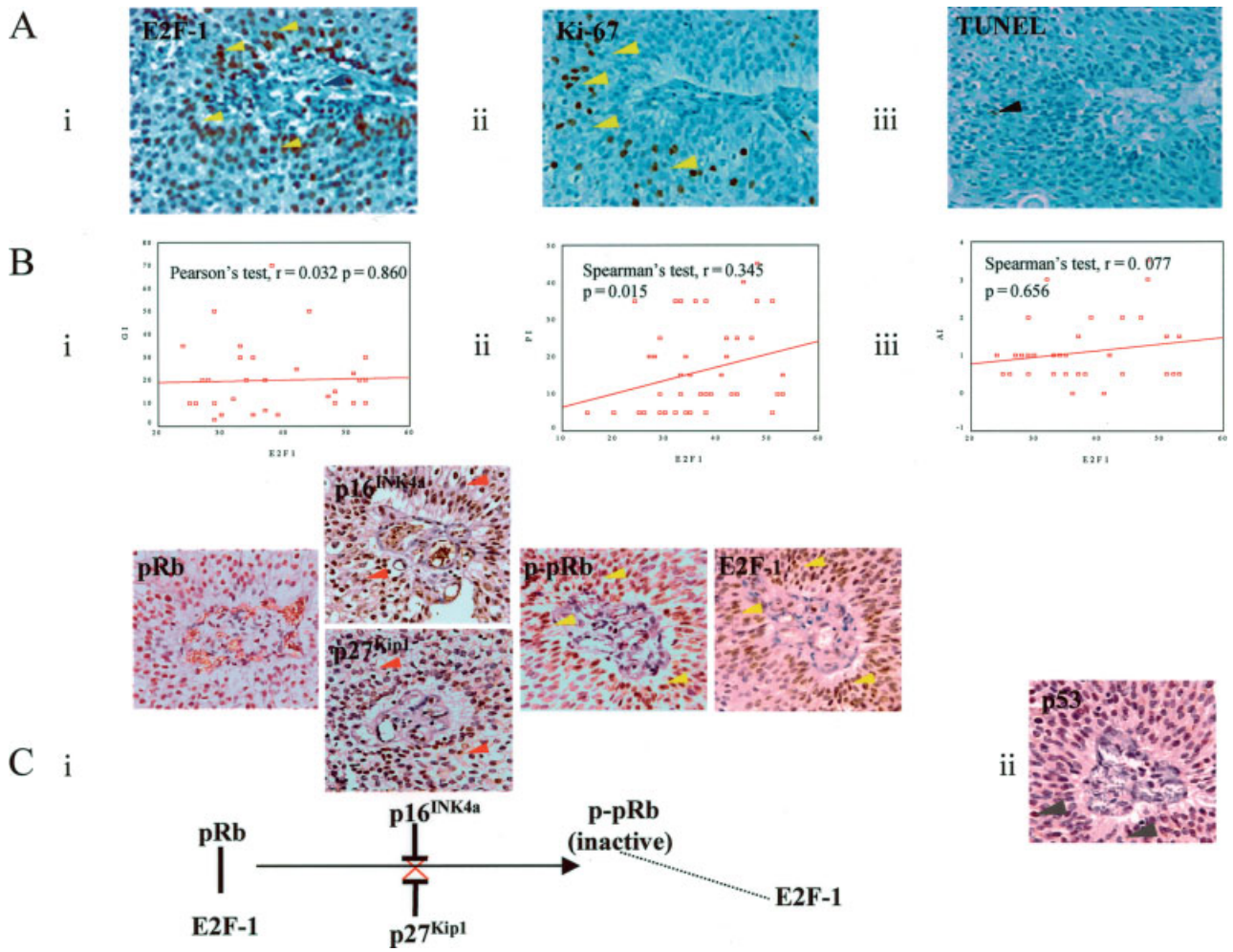


Figure 4. Relationship of E2F-1 status with tumour kinetics, pRb and p53 pathways in superficial TCC. (A) Representative results of E2F-1 and Ki-67 immunohistochemistry and TUNEL assay in serial sections. Yellow arrow-heads depict E2F-1 and Ki-67 immunostaining in tumour cells; the blue arrow-head shows E2F-1 staining in stromal cells; and the black arrow-head illustrates apoptotic nuclei stained by TUNEL assay. (B) Scatter plots and the corresponding fitted lines of the correlation between EI and GI (i), PI (ii), and AI (iii). The results of the appropriate bivariate statistical tests are also presented. (C) Representative results for pRb and p53 status. (i) pRb, phosphorylated pRb (inactive), $p27^{Kip1}$, and $p16^{INK4a}$, as well as E2F-1, were evaluated by immunohistochemistry in serial sections. Yellow arrow-heads depict phosphorylated pRb and E2F-1 staining in the tumour area; red arrow-heads show areas with aberrant $p27^{Kip1}$ (34%) and normal $p16^{INK4a}$ status. (ii) Negative p53 immunostaining (only a small number of positive nuclei marked by black arrow-heads) implying that p53 is intact

series, we detected p53 immunoreactivity in 28 cases (48.2%). p53 immunopositivity was accompanied by the presence of mutations in 23/28 (82.1%) and LOH in 14/28 (50%) cases (Table 2 and Figure 3C, ii). LOH analysis revealed loss in 19 colon carcinomas (32.7%). Overall, p53 alterations were observed in 33/58 (56.9%) of the cases. We found no association between E2F-1 expression and p53 alterations.

TCC

Taking into consideration the current proposed model of TCC carcinogenesis, according to which inactivation of CKIs is an initial and nodal event [27], we examined the status of the universal CKI inhibitor $p27^{Kip1}$ and the G1 to S-phase CKI $p16^{INK4a}$ in relation to E2F-1 status. $p27^{Kip1}$ and $p16^{INK4a}$ expression was abnormal in 37.1% and 39.7%, respectively (Figure 4C, i). At least one of the two molecules demonstrated abnormal expression in 50 cases (64.5%). We found an inverse association between abnormal $p27^{Kip1}/p16^{INK4a}$ immuno-expression and EI (38.8 ± 9.2 versus 33.5 ± 9.2, $p = 0.05$ by t-test), whereas pRb was regularly expressed in the majority of the cases. As in breast carcinomas, p-pRb immunostaining strictly paralleled E2F-1 expression (Figure 4C, i). p53 staining was absent in all but four cases (5.2%) (Figure 4C, ii). Tissue was not available for p53 sequencing analysis of these four p53-positive samples.

Discussion

The bimodal effect of E2F-1, in experimental models, on cellular kinetics is a matter of great interest and debate in carcinogenesis, and its actions in each particular type of cancer need to be elucidated. We therefore examined E2F-1 expression and its relationship with

Table 2. Mutation and allelic imbalance (AIm) analysis of the p53 immunopositive breast and colon carcinomas

		p53 alterations			
		Mutation status			
No	Case No	Codon	Point mutation	Amino acid substitution	D17S179E AIm
Breast carcinomas					
1	4	74	GCC to ACC	Ala to Thr	het
2	13	135	TGC to TAC	Cys to Tyr	ho
3	17	248	CGG to CAG	Arg to Trp	het
4	23	175	CGC to CAC	Arg to His	het
5	26	275	TGT to TAT	Cys to Tyr	ho
6	27	248	CGG to CAG	Arg to Trp	het
7	30	220	TAT to TGT	Tyr to Cys	LOH
8	33	273	CGT to CAT	Arg to His	het
9	36	245	GGC to AGC	Gly to Ser	het
10	44	175	CGC to CAC	Arg to His	het
Colon carcinomas					
1	1	175	CGC to CAC	Arg to His	LOH
2	5	248	CGG to TGG	Arg to Trp	LOH
3	8	237	ATG to ATA	Met to Ile	LOH
4	9	244	GGC to AGC	Gly to Ser	LOH
5	12	279	GGG to GTG	Gly to Glu	LOH
6	16	286	GAA to AAA	Glu to Lys	LOH
7	17	249	AGG to AGT	Arg to Ser	LOH
8	21	240	AGT to GGT	Ser to Gly	het
9	24	175	CGC to CAC	Arg to His	het
10	28	69	GCT to GGT	Ala to Gly	LOH
11	29	238	TGT to TAT	Cys to Tyr	LOH
12	34	245	GGC to AGC	Gly to Ser	het
13	35	248	CGG to CAG	Arg to Gln	ho
14	37	282	CGG to TGG	Arg to Trp	LOH
15	44	273	CGT to CAT	Arg to His	het
16	46	273	CGT to CAT	Arg to His	het
17	47	245	GGC to AGC	Gly to Ser	het
18	49	238	TGT to TAT	Cys to Tyr	LOH
19	50	179	CAT to TAT	His to Tyr	LOH
20	52	173	GTG to TTG	Val to Leu	het
21	54	175	CGC to CAC	Arg to His	LOH
22	57	248	CGG to CAG	Arg to Gln	LOH
23	58	157	GTC to TTC	Val to Phe	het

het = heterozygous, ho = homozygous, LOH = loss of heterozygosity.

growth-determining parameters in BrC, PrC, CC, and TCC, four of the most common human malignancies.

In three of the tumour types examined, namely BrC, superficial TCC, and CC, E2F-1 expression was elevated compared with their normal counterparts. The observed EI values were slightly higher than those presented in the few recent studies examining E2F-1 in the corresponding carcinomas [13,28,29]. An unexpected finding was the absence of E2F-1 expression in prostate tumours, although cytoplasmic staining was noted in some cases, probably reflecting dimerization of E2F-1 with a splice variant of DP2, known to influence E2F localization [2]. This particular immunohistochemical profile of E2F-1 in prostate carcinomas deserves further investigation, since it could possibly serve as an additional diagnostic tool in cases where diagnosis based on morphological criteria is ambiguous. Subsequently, to address the functionality of E2F-1, we examined the phosphorylation status of pRb (p-pRb) in the carcinomas with intact expression of total pRb. The high degree of co-localization found between E2F-1 and p-pRb (Figures 2C, 3C, and 4C) suggests that a significant portion of the E2F-1 detected *in situ* corresponds to the active form of the protein that is not bound to pRb. In the cases with aberrant/absent pRb, we considered that E2F-1 expression reflected the free and active fraction of E2F-1.

To investigate the putative effect of 'active' E2F-1 on the growth of the carcinomas examined, we analysed serial sections, studying E2F-1 and the kinetic parameters of the tumours concurrently. By this process, we were able to address and illustrate *in vivo* the issue of E2F-1 behaviour in various types of cancer.

Thus, in BrC and superficial TCC, E2F-1 was associated with proliferation. In the former case, elevated EI also correlated with tumour growth. However, such an association was not observed in superficial TCC, possibly mirroring the positive correlation between proliferation and apoptosis ($r = 0.322, p = 0.052$), as previously demonstrated [27]. These data are in agreement with previous reports on BrC [13], and non-invasive papillary neoplasms [30], implying that within these environments, E2F-1 acts as a growth-promoting factor. Similar behaviour of E2F-1 has been reported in lung cancer [11,12], thyroid lesions [16], and pancreatic carcinomas [17].

In the CC series, the reverse appears to apply, since E2F-1 was inversely associated with tumour growth. Analogous E2F-1 behaviour has been reported in bladder carcinomas [29] and diffuse large B-cell lymphomas [14]. The former findings in bladder cancer [29] seem at first to contradict ours. However, superficial low-grade TCC exhibits distinct molecular and kinetic features compared with invasive bladder tumours [27], which represent the majority of the cases investigated by Rabbani *et al* [29].

Our univariate survival analysis supports the relationship between E2F-1 and tumour kinetics (see the Results section). However, further clinical research on patients' outcome is required to avoid the limitations of survival analysis of the small samples of each carcinoma examined.

Finally, in PrCs, the absence of E2F-1 immunoreactivity implies either oncosuppressor activity or that its expression is dispensable for tumour growth modulation. Examining the scenario that other E2F activator members may be implicated in prostate carcinogenesis, we interestingly observed nuclear staining of prostate tumour cells for E2F-2. Furthermore, we found increased expression of E2F-2 in the PC-3 prostate carcinoma cell line, whereas E2F-1 was absent. Similarly, in myeloid cell lines, E2F-3, but not E2F-1, was found to be responsible for growth factor-dependent proliferation and differentiation [31].

The answer that justifies this cancer-dependent behaviour of E2F-1 and probably elucidates its apparent contradictory behaviour may lie in its relationship with the two major tumour suppressor routes of the cell, pRb and p53, which affect its activity directly and

indirectly [2,4]. In tumours, deregulation of the pRb pathway via pRb inactivation, decreased $p16^{INK4a}$ and $p27^{Kip1}$ levels, and high cyclin D1 and E expression is a common event leading to an increase in pRb-unrestrained E2F-1 levels [3,4,6,7]. Unleashed E2F-1 will influence the balance between proliferation and apoptosis depending mainly on the status of p53 [32]. E2F-1 contributes to proliferation if the p53 pathway is 'switched off'; otherwise, it promotes programmed cell death [2,33,34]. To examine the above scenario, we investigated in our series the relationship of E2F-1 with the status of the pRb and p53 pathways.

In BrC, the above scenario seems to apply, since increased E2F-1 expression was associated with abnormal expression of the $p16^{INK4a}/p27^{Kip1}$/pRb network ($p = 0.016$) and defective p53 status ($p = 0.042$). Our findings regarding pRb and p53 are in accordance with previous results showing that breast carcinomas exhibiting high proliferation were characterized by aberrations of the pRb and p53 pathways [35]. In superficial TCC, the same model appears to fit as well. In particular, pRb inactivation via down-regulation of CKIs, which is an early and nodal molecular event in the development of bladder superficial TCC [27], was observed in our series and was associated with increased values of EI ($p = 0.051$). However, the absence of an association with GI may be attributed to the fact that the apoptotic pathways seem to be active, a notion supported by the low frequency of p53 aberrations found (5.2%).

On the contrary, in colonic adenocarcinomas, E2F-1 release appears to be linked to activation of apoptosis, despite the high percentage of p53 alterations observed. In this case, apoptosis may be mediated by the p53 homologue p73 and apoptosis protease activating factor-1 (Apaf-1) [2,4,36]. The very low frequency of p73 alterations in colonic carcinomas [37] as well as increased Apaf-1 expression identified in human colonic cell lines [38] supports the above notion. Furthermore, it has been demonstrated, in colonic cell lines, that forced E2F-1 expression, even if associated with the G1–S transition, finally leads to G2/M cell-cycle arrest and apoptosis [38,39]. In keeping with our findings is the paradoxical progressive, and often irregular, increase in pRb expression alongside elevated proliferation that is observed during the various steps of colonic carcinogenesis, suggesting that it may be recruited to provide 'protection' from the 'deleterious' effects of E2F-1 [40].

Taking together the data presented in this study, we suggest that in human malignancies, E2F-1 exhibits a multifarious behaviour that seems to depend on the specific cellular context and the activation status of the pathways converging on it. Elucidating the role of E2F-1 in various types of cancer will help in the development of specific anti-cancer agents based on E2F biology [41]. Furthermore, as our preliminary findings in the PC-3 prostate carcinoma cell line and prostate carcinomas show, other member(s) of the E2F family may exert an important role in tumour biology and their status should be individually addressed, in a manner similar to E2F-1, in human malignancies.

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