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Loss of APAF-1 expression is associated with tumour progression and adverse prognosis in colorectal cancer

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ABSTRACT

The aim of this study was to determine the prognostic value of APAF-1 in colorectal cancer (CRC). Immunohistochemistry for APAF-1 was performed on a tissue microarray of 1015 mismatch-repair (MMR) proficient and 130 sporadic MLH1-negative CRCs. The association of APAF-1 with clinico-pathological features including 10-year survival time was analysed. Methylation specific PCR was performed on a subset of MMR-proficient and MLH1-negative CRC. Loss of APAF-1 was associated with advanced T stage (p-value = 0.022), N stage (p-value = 0.009), vascular invasion (p-value = 0.001) and worse survival (p-value = 0.017) in MMR-proficient CRC. In MLH1-negative CRC, loss of APAF-1 was associated with metastasis (p-value = 0.041), worse prognosis (p-value < 0.001) and independently predicted shorter survival time (p-value < 0.001). No methylation was found in the selected region of *APAF-1*. APAF-1 is a marker of tumour progression in MMR-proficient CRC and an independent adverse prognostic factor in MLH1-negative CRC.

1. Introduction

Apoptosis protease activating factor-1 (APAF-1) is a 130 kDa protein that plays a central role in the activation of caspases involved in mitochondria-mediated apoptosis.[1] The APAF-1 protein consists of three domains: the N-terminal caspase recruitment domain (CARD), the CED-4 like domain responsible for nucleotide binding and the C-terminal domain containing multiple repeats or tryptophan and aspartate residues (WD repeats) essential for carrying out protein–protein interactions.[1] Cytochrome *c* released from the mitochondria following apoptotic stimuli binds to the WD region of the APAF-1 protein.[2] In the presence of dATP, conformational changes of the WD region unmask the CARD domain allowing the binding of pro-caspase-9. Oligomerisation of the APAF-1 protein ensues through its CED-4 like domains creating a 7-spoke wheel-like structure called the apoptosome.[2] Subsequent activation of pro-caspase-9 by autocatalytic cleavage initiates a cascade of downstream effector caspases ultimately resulting in apoptosis.

APAF-1 is an essential component of developmental programmed cell death. Homozygous null mutant mice die *in utero* or shortly after birth and exhibit severe craniofacial abnormalities, retention of interdigital webs, as well as abnormal eye and inner ear development.[3] *APAF-1* knockouts show brain overgrowth due to hyperproliferation of neuronal cells, whereas heterozygous mice do not show these alterations. Absence of APAF-1 protein appears to prevent activation of

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caspase-3 *in vivo* and to impair processing of caspases -2 and -8 leading to cellular resistance to apoptotic stimuli.[4,5]

APAF-1 appears to act as a tumour-suppressor gene.[1] Mustika et al. described intense and diffuse cytoplasmic immunohistochemistry (IHC) staining for APAF-1 in normal skin, nevi and melanoma *in situ*.[6] Weaker, focal positivity was observed in melanoma and in less than 25% of all tumour cells from metastatic melanoma suggesting a role for APAF-1 in disease progression. Additionally, an inverse correlation between APAF-1 expression and pathologic stage has been reported in this disease.[1] Loss of heterozygosity at the *APAF-1* locus (12q22-23) has been correlated with decreased mRNA expression in metastatic melanoma as well as with poor disease outcome and chemo-resistance.[7] In colorectal cancer (CRC), an increased frequency of allelic imbalance at the *APAF-1* locus has been associated with tumour progression from adenoma to carcinoma to metastatic cancer.[8] Patients with rectal cancer undergoing pre-operative radiotherapy with decreased APAF-1 expression in the pre-treatment biopsy demonstrated a worse response to treatment than patients positive for the protein.[9]

Though the cascade of apoptotic events surrounding APAF-1 activation is well documented, the value of APAF-1 as a prognostic factor in CRC has not yet been evaluated. The aim of this study was to determine using tissue microarray (TMA) technology whether APAF-1 is a marker of tumour progression and prognosis in 1420 CRC stratified by MMR-status.

2. Materials and methods

2.1. TMA construction

A TMA of 1420 unselected CRCs and 57 normal colon tissues was constructed as described previously.[10] Formalin-fixed, paraffin-embedded tissue blocks of CRC resections were retrieved from the archives of the Institute of Pathology, University Hospital of Basel, Switzerland; the Institute of Clinical Pathology, Basel, Switzerland; and the Institute of Pathology, Stadtspital Triemli, Zürich, Switzerland. One tissue cylinders with a diameter of 0.6 mm were punched from morphologically representative tissue areas of each donor tissue block and brought into one recipient paraffin block (3×2.5 cm) using a homemade semiautomated tissue arrayer.

2.2. Clinico-pathologic data and tumours

The clinico-pathologic data for 1420 patients included T stage (T1, T2, T3 and T4), N stage (N0, N1 and N2), tumour grade (G1, G2 and G3), vascular invasion (presence or absence) and survival. The distribution of these features has been described previously.[11] For 478 patients, information on local recurrence and distant metastasis was also available.

2.3. IHC

Four-micron sections of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics, Inc., Hackensack, NJ) to facilitate the transfer of TMA sections to slides and to minimise tissue loss. Standard indirect immunoperoxidase procedures were used for IHC. 1420 CRCs and 57 normal colonic mucosa samples were immunostained for APAF-1 (clone NCL-APAF-1; dilution 1:40, Novocastra, UK). CRCs were additionally stained for MLH1 (clone MLH-1; dilution 1:100; BD Biosciences Pharmingen, San Jose, CA), MSH2 (clone MSH-2; dilution 1:200; BD Biosciences Pharmingen, San Jose, CA) and MSH6 (clone 44; dilution 1:500; BD Biosciences Pharmingen, San Jose, CA). After dewaxing and rehydration in dH2O, sections for immunostaining were subjected to heat antigen retrieval in a microwave oven (1200 W, 15 min) in 0.01 mol/l citrate buffer for APAF-1, pH 7.0, and MSH6, pH 6, and 0.001 mol/L ethylenediaminetetraacetic acid, pH 8.0, for MLH1 and MSH2. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. After transfer to a humidified chamber, the sections were incubated with 10% normal goat serum (Dako Cytomation, Carpineteria, CA) for 20 min and incubated with primary antibody at 4° overnight. Subsequently, the sections were incubated with secondary antibody (DakoCytomation) for 30 min at room temperature. For visualisation of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole + substrate-chromogen (DakoCytomation) for 30 min, and counterstained lightly with Gill's haematoxylin.

2.4. IHC evaluation

APAF-1 immunoreactivity in 1420 CRCs was determined quantitatively by one experienced pathologist (A.L.) evaluating the proportion of positive tumour cells over total tumour cells. Positivity was scored on a scale of 5% intervals (0%, 5%, 10%, etc.). In order to determine the inter-observer reproducibility of this scoring method, two additional pathologists (K.K, D.H.) also scored 454 CRCs. All 1420 tumours were re-scored (A.L.) 1 month later to determine the intra-observer reliability. Staining intensity was not evaluated. IHC for MLH1, MSH2 and MSH6 was scored (A.L.) as negative when no staining was observed and as positive when any immunoreactivity was found.

2.5. DNA mismatch-repair (MMR)-status

The 1420 CRCs were stratified by DNA MMR status and consisted of 1197 MMR-proficient tumours expressing MLH1, MSH2 and MSH6, 141 MLH1-negative tumours, and 82 presumed HNPCC cases demonstrating loss of MSH2 and/or MSH6 at any age, or loss of MLH1 at <55 years.[12] The presumed HNPCC cases were excluded from this study due to the possible statistical unreliability of their small numbers.

2.6. DNA extraction and bisulphite modification

Twenty-eight samples from MMR-proficient and MLH1-negative CRCs with various IHC expression levels of APAF-1 protein were micro-dissected from paraffin-embedded tissue using two 8-μm thick sections. Cell lysis and DNA extraction were performed using a QIAamp DNA mini kit (QIAGEN, Mississauga, ON) according to the manufacturer's protocol. Extracted genomic DNA was diluted in 40 μl of distilled water and denatured by adding 6 μl of 2 N NaOH and incubation at 75 °C for 20 min. Five-hundred microlitres of freshly prepared

4.8 M sodium bisulphite and 28 μl of 10 mM hydroquinone were added to the denatured genomic DNA and the reaction was carried out overnight in dark at 55 °C. DNA then was purified using a Wizard DNA clean-up (Promega, Madison, WI) and then ethanol-precipitated after 5 min of alkali treatment with 8.8 μl of 2 N NaOH at room temperature.

2.7. Methylation of APAF-1

Methylation of the *APAF-1* promoter was examined by methylation-specific PCR (MSP) using AmpliTaq Gold kit (Roche, Branchburg, NJ) as described previously.[13] The primers for amplification of unmethylated sequence were 5′-GATTTGAGGTGTTGTAGTGGTATTT-3′ and 3′-AAAAAAAATCTTCCCAACCTATAACA-5′ and primer sequences for methylated reaction were 5′-GAGGTGTCGTAGCGGTATTC-3′ and 3′-AAAAATCTTCCCGACCTATAACG-5′. The condition for amplification was 10 min at 95 °C followed by 39 cycles of denaturing at 95 °C for 30 s, annealing at 56 °C for 30 s and 30 s of extension at 72 °C. PCR products were subjected to electrophoresis on 8% acrylamide gels and visualised by SYBR gold nucleic acid gel stain (Molecular Probes, Eugene, USA). CpGenome Universal Methylated DNA (Chemicon, Temecula, CA) was used as a positive control for methylation.

2.8. Statistics

2.8.1. Intra- and inter-observer reliability of the scoring method

The inter-observer reliability of the scoring method was evaluated using the intra-class correlation coefficient (ICC). The ICC defined as the ratio of the between-subject variance over the (between-subject + within subject variances) has previously been used to assess agreement of IHC scores.[14] The intra-observer agreement was found using Cronbach's alpha (∀) statistic. Alpha values greater than 0.8 suggest excellent consistency of scores.

2.8.2. Selection of cut-off scores for APAF-1 positivity

In order to determine the cut-off score for positive APAF-1 expression, receiver operating characteristic (ROC) curve analysis was used.[15] Clinically, relevant cut-off scores were selected such that they maximised both sensitivity and specificity for each outcome under study.[16] In order to apply ROC curve analysis, the clinico-pathologic features were dichotomised as follows: early (T1 + T2) or late (T3 + T4) T stage, no (N0) or any (>N0) lymph node involvement, low (G1 + G2) or high (G3) tumour grade, absence or presence of vascular invasion, 10-year disease-specific survival time (death due to CRC versus death from other causes or lost to follow-up), absence or presence of local recurrence and absence or presence of metastasis. The reliability of the cut-offs was determined using 100 bootstrap replications of the data.

2.8.3. Association of APAF-1 and clinico-pathologic features

The association of APAF-1 with T stage, N stage, tumour grade, vascular invasion, recurrence and metastasis was determined using the Chi-Square or Fisher's Exact test. Univariate survival analysis was performed using the Kaplan–Meier method and multivariate analysis by Cox proportional hazards regression. The hazard ratios (HR) and 95% confidence intervals (CI) were obtained. All analyses were carried out using SAS (Version 9.1, The SAS Institute, NC, USA).

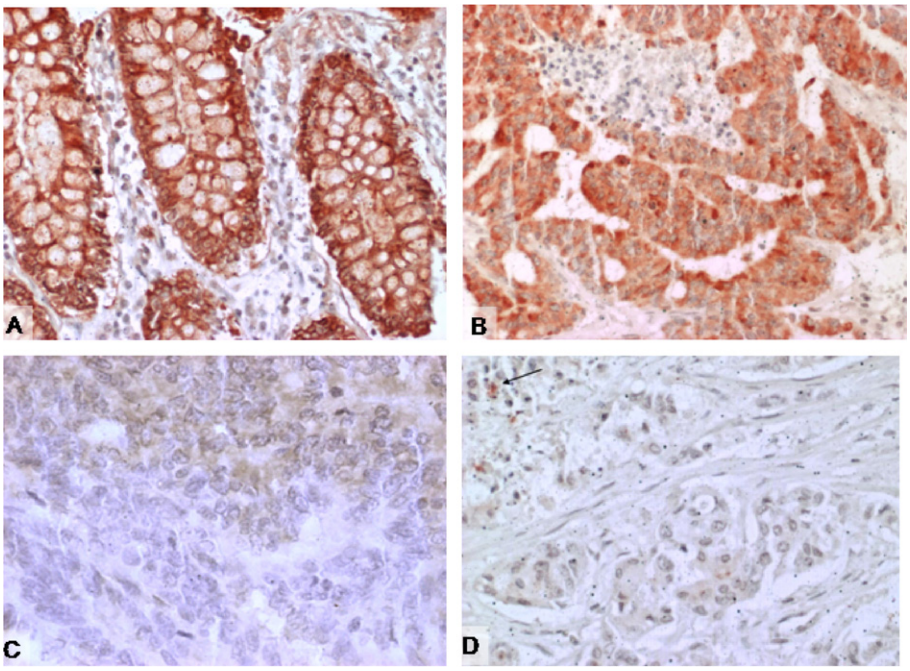

Fig. 1 – Strong cytoplasmic expression of APAF-1 in normal colonic mucosa (A) and in a moderately differentiated MMR-proficient CRC (B) (40×). Partial loss (C) and complete loss (D) of APAF-1 expression in a moderately differentiated MMR-proficient CRC (40×) (arrow: APAF-1 positive inflammatory cell as internal positive control).

Table 1 – Association of APAF-1 and clinico-pathological features in MMR-proficient tumours

	Loss of APAF-1		No loss of APAF-1		*p*-Value
	N	(%)	*N*	(%)	
T stage					
T1	29	(4.9)	15	(3.7)	0.022
T2	79	(13.4)	84	(20.6)	
T3	391	(66.4)	251	(61.7)	
T4	90	(15.3)	57	(14.0)	
N stage					
N0	271	(46.9)	227	(56.9)	0.009
N1	171	(29.6)	97	(24.3)	
N2	136	(23.5)	75	(18.8)	
Tumour grade					
G1	14	(2.4)	6	(1.5)	0.403
G2	523	(88.6)	357	(87.7)	
G3	53	(9.0)	44	(10.8)	
Vascular invasion					
Presence	197	(33.4)	96	(23.5)	0.001
Absence	393	(66.6)	312	(76.5)	
Metastasis					
Yes	41	(43.2)	78	(38.2)	0.418
No	54	(56.8)	126	(61.8)	
Local recurrence					
Yes	45	(47.9)	94	(46.8)	0.859
No	49	(52.1)	107	(53.2)	
Median survival (95% CI) months	60.0 (49.0–71.0)		85.0 (63.0–104.0)		0.017

3. Results

3.1. *Inter- and intra-observer reliability of APAF-1 scores*

The ICC was 0.75 indicating strong inter-observer agreement of APAF-1 scores between three pathologists. The intra-observer agreement was 0.87 suggesting excellent consistency of scores.

3.2. *Cut-off scores for APAF-1 positivity*

The cut-off scores for APAF-1 positivity were 90% for T stage, N stage, vascular invasion, presence of metastasis and local recurrence and 100% for tumour grade and survival. Loss of APAF-1 was defined as a score below the respective cut-off score, while no loss was characterised as APAF-1 positivity above or equal to the score.

3.3. *APAF-1 expression in normal tissue and CRC*

All 57 normal tissue samples were immunoreactive for APAF-1 in 100% of cells (Fig. 1A). MMR-proficient CRCs (1015) were available for analysis of APAF-1 expression. Of these 416 (41.0%) CRCs demonstrated the expression of APAF-1 in 100% of tumour cells. Of the 130 MLH1-negative CRCs which could be evaluated for APAF-1 expression, 84 (64.6%) had 100% tumour cell staining (Fig. 1B–D). This difference in APAF-1 expression across MMR groups was found to be significant (p-value < 0.001).

3.4. *MMR-proficient CRC and APAF-1 expression (Table 1)*

APAF-1 expression was significantly associated with T stage (p-value = 0.022), specifically with differences in T2 and T3 CRC. Loss of APAF-1 was more frequently found in CRCs with lymph node involvement (p-value = 0.009) and with vascular invasion (p-value = 0.001). There was no association between APAF-1 and tumour grade, the presence of distant metastasis or local recurrence (see Table 1).

3.5. *Survival analysis in MMR-proficient CRC*

The median 10-year survival time for patients with loss of APAF-1 expression was 60.0 (49.0–71.0) months; patients with no loss of APAF-1 had significantly better prognosis (85.0 (63.0–104.0) months; p-value = 0.017) (Fig. 2). Loss of APAF-1 expression resulted in lower 5-year and 10-year survival rates (50.0% and 33.7%, respectively) compared to patients with no loss of APAF-1 expression (55.5% and 40.1%, respectively). APAF-1 was not an independent indicator of prognosis (p-value = 0.3569; HR (95% CI) = 0.913 (0.75–1.11)) in a multivariate analysis adjusting for T stage, N stage, tumour grade, vascular invasion and age.

3.6. *MLH1-negative CRC and APAF-1 expression (Table 2)*

APAF-1 expression was significantly associated with the absence of distant metastasis (p-value = 0.041) and was linked to local recurrence (p-value = 0.053) though only

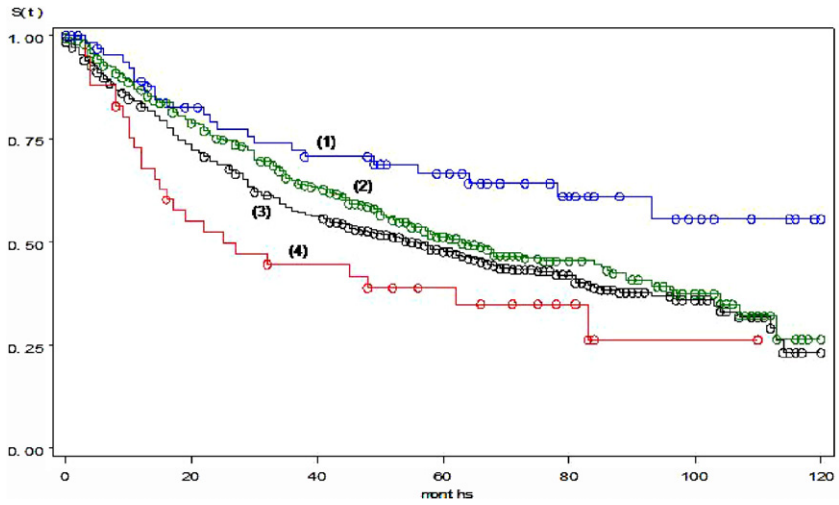

Fig. 2 – Kaplan–Meier 10-year survival curves for: (1) MLH1-negative CRC with no loss of APAF-1, (2) MMR-proficient CRC with no loss of APAF-1, (3) MMR-proficient CRC with loss of APAF-1, and (4) MLH1-negative CRC with loss of APAF-1.

Table 2 – Association of APAF-1 and clinico-pathological features in MLH1-negative tumours

	Loss of APAF-1		No loss of APAF-1		*p*-Value
	N	(%)	N	(%)	
T stage					
T1	0	(0.0)	0	(0.0)	0.659
T2	2	(4.4)	4	(4.8)	
T3	35	(76.1)	57	(68.7)	
T4	9	(19.6)	22	(26.5)	
N stage					
N0	21	(45.7)	52	(63.4)	0.066
N1	10	(21.7)	17	(20.7)	
N2	15	(32.6)	13	(15.9)	
Tumour grade					
G1	0	(0.0)	2	(2.4)	0.343
G2	37	(80.4)	58	(70.7)	
G3	9	(19.6)	22	(26.8)	
Vascular invasion					
Presence	13	(28.3)	19	(23.5)	0.549
Absence	33	(71.7)	62	(76.5)	
Metastasis					
Yes	9	(50.0)	14	(24.6)	0.041
No	9	(50.0)	43	(75.4)	
Local recurrence					
Yes	8	(44.4)	17	(30.4)	0.053
No	10	(55.6)	39	(69.6)	
Mean survival time (months)	41.4 ± 5.4		68.2 ± 4.5		<0.001

marginally. APAF-1 expression was not related to T stage, N stage, tumour grade or vascular invasion (see Table 2).

3.7. *Survival analysis of MLH1-negative CRC*

Loss of APAF-1 expression was associated with a significantly lower mean 10-year survival time (44.4 ± 5.4 months) compared to no loss of the protein (68.2 months ± 4.5) (p-value < 0.001) (Fig. 2). The 10-year survival rate for patients with loss of APAF-1 was 39.5% compared to 69.3% for patients with no loss of expression. Multivariate survival analysis demonstrated that loss of APAF-1 was associated with worse survival time (p-value < 0.001; HR (95% CI) = 0.45 (0.25–0.82)) independently of T stage, N stage, tumour grade, vascular invasion and age.

3.8. Methylation analysis

Neither MMR-proficient nor MLH1-negative CRCs were found to have methylation of the promoter region of *APAF-1*.

4. Discussion

Recent advances in the understanding of cell death pathways activated by anticancer drugs and radiation have led to the development of novel apoptosis-directed therapies.[17–19] Mediators of mitochondria-dependent apoptosis including Bcl-2 family members, p53, caspases and inhibitors of apoptosis proteins (IAP) have been shown to modulate the effects of both chemotherapeutic agents and radiotherapy and are currently being explored as potential targets for therapeutic intervention.[20–22] The APAF-1 protein constitutes the central element of the mitochondrial death machinery.[1] Though the molecular events surrounding the activation of APAF-1 are well characterised, few studies have investigated the role of APAF-1 in colorectal tumour progression.[8] In addition, the prognostic value of the protein in CRC has not yet been established.

In this study, IHC for APAF-1 was carried out on MMR-proficient ($N = 1015$) and sporadic MLH1-negative ($N = 130$) CRCs with complete clinico-pathological data and 10-year follow-up. Immunostaining on this large number of tissues was performed using TMA technology.[23] APAF-1 immunoreactivity was assessed using a scoring system based on the percentage of positive tumour cells. We have recently shown that this evaluation method is reproducible and leads to a more complete assessment of the prognostic or predictive value of several tumour markers in CRC over a method using a predetermined and often arbitrary cut-off score to classify 'positive' or 'negative' tumours.[24,25] The reliability of this scoring system for APAF-1 was evaluated in this study by three pathologists and was found again to be highly reproducible.

In order to select clinically relevant IHC cut-off scores for APAF-1 positivity, ROC curve analysis was performed for each of the clinico-pathological features.[15] This method has previously been used in CRC.[16] The cut-off score for T stage, N stage, vascular invasion, presence of metastasis and local recurrence was 90%, whereas that for tumour grade and survival was 100%. These cut-off scores are consistent with the literature describing APAF-1 as a putative tumour-suppressor gene.[1]

In MMR-proficient CRC, a significant difference in APAF-1 expression was observed for T stage, N stage and vascular invasion. Patients with loss of APAF-1 had a significantly worse prognosis than those retaining complete expression of the protein. However, in multivariate analysis APAF-1 expression was not found to have independent prognostic value. Loss of APAF-1 expression was linked to the presence of metastasis in MLH1-negative CRC and correlated with worse survival time compared to tumours with complete expression of the protein. Loss of APAF-1 expression was found to be an independent adverse prognostic factor in MLH1-negative CRC.

Our results are in line with numerous findings in malignant melanoma that identified loss of APAF-1 as a key feature in tumour progression.[6,26,27] An inverse correlation between APAF-1 expression and pathologic stage has been reported.[6] Moreover, the IHC expression of APAF-1 was found to be significantly lower in metastatic melanomas compared to tumours with no metastases.[26] Reduced APAF-1 expression was associated with more advanced nodal stage in cervical cancer.[28] Allelic imbalance at the APAF-1 locus was found to correlate with the progression of colorectal tumours.[8]

Numerous recent studies have shown that promoter hypermethylation leads to loss of *APAF-1* in several cancer types, including melanoma and renal cell carcinoma.[29,30] In order to verify whether methylation was responsible for inactivation of *APAF-1* in CRC, we performed methylation specific PCR on 28 cases of MMR-proficient as well as MLH1-negative CRC demonstrating IHC expression for APAF-1 ranging from 0% to 100%. No methylation was found in the selected region of *APAF-1*.

In MMR-proficient CRC, APAF-1 plays a role in tumour progression and survival. Loss of APAF-1 expression is a marker of adverse outcome in MLH1-negative CRC, and appears to affect survival time independently of known prognostic indicators.

Conflict of interest statement

The authors have no conflict of interest to declare.

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