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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