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Comprehensive expression analysis of TNF-related apoptosis-inducing ligand and its receptors in colorectal cancer: Correlation with MAPK alterations and clinicopathological associations

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ABSTRACT

TNF-related, apoptosis-inducing ligand (TRAIL) apoptotic pathway constitutes a promising therapeutic target due to high selectivity and low toxicity of TRAIL targeting agents when administered in combination therapies. 106 colorectal cancers were examined for: relative mRNA expression of TRAIL pathway genes, decoy receptors TRAIL-R3 and TRAIL-R4 promoter methylation and the presence of KRAS, NRAS, BRAF mutations. Elevated mRNA levels were observed in 26%, 15%, 13%, 12% and 10% of the cases for TRAIL-R4, TRAIL-R3, TRAIL-R2, TRAIL-R1 and TRAIL genes respectively. Reduced mRNA levels were detected in 77%, 65%, 64%, 60% and 37% of the cases for TRAIL, TRAIL-R2, TRAIL-R3, TRAIL-R1 and TRAIL-R4 genes respectively. TRAIL-R3 and TRAIL-R4 promoter methylation was detected in 55% and 16% of the analysed samples respectively. TRAIL-R1, TRAIL-R2 elevated relative mRNA levels inversely correlated with tumor stage ($p = .036$, $p = .048$). Strong linear correlations of TRAIL receptors' mRNA levels were found: TRAIL-R1/TRAIL-R2 ($R = 0.653$, $p < .001$), TRAIL-R2/TRAIL-R3 ($R = 0.573$, $p < .001$). Finally, relative expression of TRAIL was correlated with KRAS, BRAF and NRAS mutation status, defining an inverse correlation between increased TRAIL expression and the absence of mutations in Mitogen-activated protein kinase (MAPK) pathway.

In conclusion, simultaneous analysis of TRAIL pathway membrane components, pointed towards a significant deregulation of mRNA expression in colorectal tumours. Death receptor overexpression was an indicator of a less aggressive phenotype. The multiple expression patterns of TRAIL pathway components in colorectal tumours underscore the importance of patient selection in order to achieve maximum efficiency with TRAIL targeted therapy.

1. Introduction

Apoptosis (programmed cell death) is implicated in various physiological and developmental processes, serving as a natural barrier to cancer development. Perturbations in apoptotic cell death regulation are considered as a step of crucial importance in cancer evolution. Tumour necrosis factor Related Apoptosis Inducing Ligand (TRAIL) pathway figures prominently as a major cell signaling pathway for the execution of apoptosis. TRAIL pathway participates in significant physiological cell functions such as cancer immunosurveillance and metastasis control [1,2].

TRAIL is a type II transmembrane protein that belongs to tumour necrosis factor (TNF) superfamily. It activates apoptosis through binding as a trimer its two agonistic cognate death receptors TRAIL-R1 and TRAIL-R2 which carry intracellular cytoplasmic death domains (DD) [3]. The intracellular receptor DDs take part in the formation of Death Inducing Signaling Complex (DISC) with the recruitment of Fas Associated protein with Death Domain (FADD) and pro-caspase 8 initiator caspase, culminating in the activation of the intrinsic apoptotic pathway triggering selective neoplastic cell apoptosis [4]. TRAIL binds also to decoy receptors, namely TRAIL-R3 and TRAIL-R4, lacking an intracellular death domain, thus impairing apoptosis. These decoy receptors have been referred to as competitive inhibitors preventing TRAIL induced apoptotic cell death [5].

TRAIL pathway is also known to interact with other signaling cascades commonly involved in carcinogenesis such as RAS/RAF/MEK/

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ERK, which is related to cell proliferation and differentiation. RAS mutations are common events in colorectal cancer and have been associated with expression of TRAIL pathway genes [6,7] leading to proliferation rather than to apoptosis induction [8]. TRAIL pathway constitutes a promising therapeutic target due to high selectivity and low toxicity of TRAIL targeting agents (e.g. monoclonal antibodies, recombinant ligand molecules, natural products) when administered in combination therapies as shown in clinical trials [9]. The most important impediment for an effective TRAIL targeted therapy remains the development of resistance, including MAPK pathway activation and overexpression of decoy receptors as candidate resistance factors [10].

Although TRAIL pathway component expression is widely viewed as a potential predictive or prognostic biomarker, there are still conflicting results surrounding its clinical importance, the levels of expression of the four receptors, as well as their expression patterns in specific tumour types [11]. Moreover, no study has yet addressed the issue of concurrent expression of death decoy receptors and TRAIL at the mRNA level, in order to define specific expression patterns associated with colorectal cancer and their significance for tumour aggressiveness. Additionally, little is known concerning the implications of TRAIL pathway gene promoter methylation status in colorectal tumorigenesis [12,13].

In the present study, we analysed the relative mRNA expression levels of TRAIL along with its death/decoy receptors, TRAIL R3/TRAIL-R4 promoter methylation status and current colorectal cancer biomarkers, such as MAPK pathway mutations, in colorectal carcinomas in the presence of standard clinicopathological prognosticators. Our aim was to assess TRAIL pathway components' concurrent expression patterns and clinicopathological associations in an effort to determine their implication in colorectal tumour aggressiveness and therefore their possible prognostic significance.

2. Materials and methods

2.1. Patients

This is a retrospective study of 106 fresh frozen consecutive cases of primary colorectal adenocarcinoma (62 males, 44 females, median age 70, age range 37–87). The present study was approved by the University of Athens Ethics Committee (Protocol No.5834/19-2-2015). Since this was a retrospective study the Ethics Committee waived the need for an informed consent, and a policy of strict anonymity and confidentiality was assured. All samples were anonymized and de-identified in a confidential manner before the study. All cases were diagnosed and treated at the Hippocration Hospital of Athens, Greece. None of the patients had received chemotherapy or radiation before surgery. Our cohort consisted of 92 conventional adenocarcinomas and 14 mucinous adenocarcinomas (Tables 1a). All cases were reviewed according to the WHO classification (2010) of colorectal carcinoma. In order to assign a grade of differentiation in mucinous carcinomas, MSI status was evaluated by molecular analysis of sensitive mononucleotide MSI markers (BAT25, BAT26, NR24, NR21) and confirmed by analysis of MMR protein expression. 5 cases were classified as MSI-high and low grade carcinomas, whereas the rest were classified as MSI-Low high-grade carcinomas (9 cases). According to the TNM system of cancer staging adopted by AJCC (8th Edition), patients were classified as: Stage I = 13 carcinomas, Stage II = 39 (4 cases IIA and 35 cases IIB), Stage III = 51 (2 cases as IIIA, 41 cases as IIIB and 8 cases as IIIC) and 3 cases as Stage IV. Adenocarcinoma grading was also defined: well differentiated,G1 = 2, moderately differentiated, G2 = 62, poorly differentiated, G3 = 28.

2.2. RNA/DNA isolation

RNA was extracted from 106 fresh tumours, preserved in RNA*later*™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 10 healthy donor blood samples using TRIzol® (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturers' protocol. 250 ng RNA were converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA was extracted from 53 samples with remaining tissue using NucleoSpin® Tissue kit (MachereyNagel, Düren, Germany) as specified by the manufacturer (Table 1b).

Table 1a
Clinicopathological characteristics of the examined colorectal tumours.

	Total number: 106
Characteristic	
Age, Median (range)	70 (37–87 years)
Gender, Female/Male	44/62
Stage	
I	13
II	39
IIA	4
IIB	35
III	51
IIIA	2
IIIB	41
IIIC	8
IV	3
Histopathololological type	
Conventional Adenocarcinoma	92
Mucinous Adenocarcinoma	14
Grade, differentiation	
Conventional Adenocarcinoma grading	
Well differentiated, G1	2
Moderately differentiated, G2	62
Poorly differentiated, G3	28
Mucinous Adenocarcinoma grading	
MSI-H, Low grade	5
MSI-L, High grade	9

2.3. RT-PCR detection of TRAIL pathway gene relative mRNA levels

Relative mRNA expression was determined by real-time RT-PCR relative quantification on a LightCycler® 480 System (Roche Diagnostics, Roche, Basel, Switzerland) using Maxima® SYBR Green (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Relative mRNA expression level was measured by quantitative RT-PCR, on LightCycler® 480 System (Roche Diagnostics GmbH, Germany) using

Table 1b
Clinicopathological characteristics of the examined colorectal tumours for MAPK mutations.

	Total number: 53
Stage	
I	7
II	23
IIA	3
IIB	20
III	21
IIIA	1
IIIB	17
IIIC	3
IV	2
Histopathololological type	
Conventional Adenocarcinoma	47
Mucinous Adenocarcinoma	6
Grade, differentiation	
Conventional Adenocarcinoma grading	
Well differentiated, G1	2
Moderately differentiated, G2	29
Poorly differentiated, G3	16
Mucinous Adenocarcinoma grading	
MSI-H, Low grade	2
MSI-L, High grade	4