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EPAS1 mRNA in plasma from colorectal cancer patients is associated with poor outcome in advanced stages

N. MOHAMMED, M. RODRIGUEZ, V. GARCIA, J.M. GARCIA, G. DOMINGUEZ, C. PEÑA, M. HERRERA, I. GOMEZ, R. DIAZ, B. SOLDEVILLA, A. HERRERA, J. SILVA and F. BONILLA

Department of Medical Oncology, University Hospital Puerta de Hierro Majadahonda, E-28222 Madrid, Spain

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Abstract. The presence of free nucleic acids in plasma has been detected in cancer patients and is associated with poor prognosis. In the present study, the mRNA levels of three genes (*EPAS1*, *KIAA0101* and *UBE2D3*) in plasma from colorectal cancer patients were analyzed. These genes were selected from a previous study of genomic profiles, discriminating between healthy controls and colorectal cancer patients. mRNA levels were analyzed by real-time PCR in the plasma of 154 patients with colorectal cancer. The association of plasma mRNA levels with clinicopathological parameters and patient survival were analyzed. High levels of *EPAS1* in the plasma were associated with patients aged over 50 years, relapse of disease and patient mortality. When patients were divided into two groups, early (I and II) and advanced (III and IV) stages, an association was observed between high levels of *EPAS1* mRNA and worse disease-free and overall survival in advanced stages. The expression of *KIAA0101* and *UBE2D3* was not associated with poor prognosis. Thus, our results suggest that *EPAS1* mRNA levels may be an indicator of poor prognosis in colorectal cancer patients at advanced stages, obtained by a non-invasive method.

Introduction

Colorectal cancer is one of the most common malignancies in Western countries and is the second most common cause of cancer mortality. Early detection, prediction of recurrence during the pre-symptomatic phase of the disease and response to chemotherapy are all factors that improve survival. Molecular techniques and new methods of detecting metastasis or recurrent disease in these pre-clinical or pre-symptomatic phases of the disease may contribute to this strategy. However, the majority of these methods have limited sensitivity or specificity, or entail invasive procedures (1). In recent years, circulating nucleic acids in the plasma of cancer patients have been studied as a source of tumor information obtained by non-invasive methods.

The presence of extracellular nucleic acids in human plasma or serum has been well established (2). In a number of studies, higher concentrations of circulating nucleic acids were detected in the plasma or serum of cancer patients as compared to that of healthy subjects, as well as higher levels in patients with metastases than in those with localized disease (3,4). Alterations in DNA found in plasma from patients with diverse types of cancer were similar to alterations found in primary tumors, suggesting that plasma and serum nucleic acids originate in tumor cells (2). More recently, the extraction of RNA from the plasma of cancer patients and its subsequent analysis by reverse transcription-polymerase chain reaction (RT-PCR) was reported (2). The potential use of plasma RNA for the analysis of cancer is highly attractive for a number of reasons: it only requires a minimally invasive method (extraction of a small amount of blood); it can be obtained repeatedly and at any time during tumor progression, allowing for an analysis of treatment response; and its simplicity makes it suitable for use in the asymptomatic population at risk. In previous studies, a correlation was noted between circulating tumor cells and circulating tumor mRNA in colon cancer (5), and it was found that mRNA is more sensitive than DNA in the plasma of breast cancer patients (6). Extracellular RNA exists with sufficient integrity in RT-PCR amplification, making it a useful tool in determining nucleic acid in the plasma of cancer patients and assessing its value as a prognostic factor. Thus, tumor-associated mRNA in plasma from cancer patients was also detected in various studies and associated with more aggressive tumors and with poor outcome (5,7-11).

In a 2007 study, cDNA microarray hybridization was employed to perform genomic profiling of plasma RNA from colorectal cancer (CRC) patients and from healthy donors (12). Expression analysis identified 40 mRNA differentially up-regulated genes in the cancer group. Using real-time PCR on a short external set of CRC samples, higher levels of the three genes (*KIAA0101*, *UBE2D3* and *EPAS1*) were confirmed in CRC patients as compared to healthy donors. Thus, these mRNA may be diagnostic indicators. *KIAA0101*, also known

Correspondence to: Dr J. Silva and Dr F. Bonilla, Department of Medical Oncology, University Hospital Puerta de Hierro Majadahonda, C/Joaquín Rodrigo, 2 E-28222 Majadahonda, Madrid, Spain
E-mail: jsilval.hpth@salud.madrid.org; fbonilla.hpth@salud.madrid.org

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as *p15PAF*, was identified as a commonly overexpressed gene in a variety of solid tumors by two independent groups using large-scale meta-analysis of cancer DNA microarray data (13,14). *EPAS1* encodes hypoxia inducible factor 2-α, an important angiogenic factor whose high expression in CRC was shown to play a significant role in tumor progression and to possess prognostic value (15). *UBE2D3*, another of our selected markers, encodes an ubiquitin-conjugating enzyme involved in the regulated degradation of major cellular factors, such as tumor suppressor p53 and NF-κB regulator (16,17).

The present study aimed to analyze the mRNA levels of the three genes (*KIAA0101*, *UBE2D3* and *EPAS1*) by real-time PCR in the plasma of a large series of patients with CRC to identify their prognostic value, and investigate potential associations with parameters indicative of poor prognosis, disease-free survival (DFS) and overall survival (OS).

Table I. Sequences and annealing temperatures (aT) for each primer used.

mRNA	Sequence	aT (˚C)
EPAS1	5'-ACGCCACCCAGTACCAGGA3'-F 5'-AATGAGGGCCCGAGCAGC3'-R	65
KIAA0101	5'-AGGTTGTCCCCTAAAGATTCTG3'-F 5'-CAGGTTGCAAAGGACATGC3'-R	59
UBE2D3	5'-AACCCAGATGACCCCCTAGTG3'-F 5'-CCATTCCCGAGATATTCTGTTG3'-R	63
SDHA	5'-TGGGAACAAGAGGGCATCTG3'-F 5'-CCACCACTGCATCAAATTCATG3'-R	59

Materials and methods

Plasma samples and clinicopathological parameters. Informed consent was obtained from all participants after an explanation of the nature of the study, as approved by the research ethics board of our hospital. All of the patients were considered sporadic cases on the basis that no clinical background of family adenomatous polyposis was reported. Any patient who met the clinical criteria for hereditary non-polyposis colon cancer (Amsterdam criteria) was excluded from the present study. Between February 2003 and January 2009, blood samples were obtained by venepuncture from 154 patients with CRC prior to intervention on the day of surgery. Plasma samples were obtained by centrifugation of peripheral blood at 500 g for 25 min and divided into aliquots, which were snap-frozen at -80˚C until processing.

The following variables were obtained from the medical record of 154 patients: age, gender, tumor site, tumor differentiation, lymph node metastases, pathological stage, histological grade, vascular invasion and evidence of polyps (defined by the presence of polyps removed during surgery). The pathological stage was assessed using the tumor-node-metastases (TNM) classification.

Patient follow-up. Clinical follow-up after surgery and diagnosis was based on periodic visits (every 3 months during the first year, every 6 months during the second year and then annually until relapse in our Medical Oncology Department, complemented by other periodic controls in Health Centers attached to our hospital), as well as clinical, biochemical and imaging techniques (chest X-ray, endoscopy, bone scan and other areas as clinically indicated). In addition, an ultrasonic study was performed when liver function was impaired. OS was defined as the period from time of diagnosis until patients succumbed to the disease. DFS was defined as the interval between diagnosis and first recurrence.

Nucleic acid isolation and real-time PCR. Plasma mRNA was obtained from 1 ml of the samples by Dynabeads mRNA direct kit (Invitrogen Dynal AS, Oslo, Norway). Plasma was incubated with 100 µl of Dynabeads oligo (dt) for 10 min at room temperature. mRNA was eluted in 10 mM HCL. For the synthesis of cDNA, RNA was retro-transcribed using the Gold RNA PCR core kit (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Random hexamers were used as primers for cDNA synthesis.

Real-time PCR was performed in a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart DNA Master^{PLUS} SYBR-Green I kit (Roche Diagnostics) according to the manufacturer's instructions. The primers and annealing temperatures are shown in Table I. The relative concentrations of the target and the reference genes were calculated by interpolation, using a standard curve of each gene plotted from the same serial dilution of cDNA from tumor tissue. The target mRNA levels (*KIAA0101*, *UBE2D3* and *EPAS1*) were normalized by the housekeeping gene, succinate dehydrogenase complex subunit A (*SDHA*), in each sample. At the end of PCR cycles, melting curve analysis and electrophoresis of the products on non-denaturing 8% polyacrylamide gels together with a molecular weight marker were performed to confirm the generation of the specific PCR product expected. Representative bands were sequenced in an ABI Prism™ 377 DNA sequencer apparatus (PE Applied Biosystems).

Statistical analysis. The target mRNA data in the plasma were not normally distributed (Kolmogorov-Smirnov test). Plasma samples showing the presence of mRNA for each target gene were divided into tertiles. The clinicopathological parameters were contrasted with the tertiles using the Pearson Chi-square test. For the survival analysis, any patients at pathological stage IV were not included in the DFS evaluation. The relationship between the cumulative probability of OS and DFS, as well as analyzed predictors, was calculated by the Kaplan-Meier method, while significant differences between curves were evaluated using the Mantel's log-rank test. In all statistical tests, two-tailed p-values <0.05 were considered to be statistically significant. Statistical analysis was performed using SPSS, version 14.0.

Results

Relationship between KIAA0101, EPAS1 and UBE2D3 mRNA levels in plasma and clinicopathological parameters. The mRNA levels of *EPAS1*, *KIAA0101* and *UBE2D3* genes were analyzed by Q-RT-PCR in a large series of 154 plasmas from

Table II. Associations between clinicopathological characteristics and mRNAs levels in the plasma from colorectal cancer patients (Chi-square test).

	KIAA0101				*EPAS1*				*UBE2D3*			
	Low (%)	Medium (%)	High (%)	p-value	Low (%)	Medium (%)	High (%)	p-value	Low (%)	Medium (%)	High (%)	p-value
Age												
<50 years	100.0	0.0	0.0	0.353	100.0	0.0	0.0	0.012	42.9	42.9	14.2	0.501
≥50 years	31.7	33.3	35.0		29.0	34.5	36.7		31.2	32.8	36.0	
Gender												
Male	35.0	27.5	37.5	0.503	33.3	31.6	35.1	0.963	32.9	31.6	35.5	0.959
Female	29.2	41.7	29.2		31.6	34.2	34.2		32.2	33.9	33.9	
Localization												
Rectum	12.5	37.5	50.0	0.418	25.0	43.8	31.3	0.127	25.0	45.0	30.0	0.081
Left	35.5	38.7	25.8		31.1	35.5	33.3		26.2	42.6	31.2	
Right	36.8	21.1	42.1		42.9	10.7	46.4		36.6	17.1	46.3	
VI												
Yes	29.4	24.0	47.1	0.357	24.1	41.4	34.5	0.353	24.3	35.1	40.6	0.349
No	34.7	36.7	29.0		37.7	29.0	33.3		37.0	32.0	31.0	
Tumor size												
<3 cm	42.8	14.3	42.8	0.603	50.0	16.6	33.3	0.699	23.1	30.8	46.1	0.677
≥3 cm	37.8	32.4	29.7		34.8	30.4	34.8		31.8	34.1	34.1	
LNM												
Yes	26.0	26.0	48.0	0.140	32.7	30.6	37.0	0.886	29.6	33.8	36.6	0.755
No	37.8	37.8	24.3		32.6	35.0	32.6		35.4	32.3	32.3	
TD												
Well	33.3	33.3	33.3	0.684	32.6	42.0	25.8	0.449	30.2	44.2	25.6	0.463
Moderate	40.0	30.0	30.0		32.0	27.7	40.4		36.2	27.6	36.2	
Poor	12.5	50.0	37.5		45.5	36.4	18.2		31.0	31.0	38.0	
Stage												
I	41.7	33.3	25.5	0.459	36.4	55.0	9.1	0.498	46.7	33.3	20.0	0.629
II	37.5	37.5	25.0	0.291[a]	32.4	29.4	38.2	0.959[a]	31.1	33.3	35.6	0.893[a]
III	25.0	20.0	55.0		35.9	30.8	33.3		26.9	38.5	34.6	
IV	28.6	43.0	28.6		20.0	30.0	50.0		36.8	21.1	42.1	
Relapse												
Yes	23.5	41.2	35.3	0.556	16.1	42.0	42.0	0.045	27.3	38.6	34.1	0.553
No	37.8	31.1	31.1		42.0	29.0	29.0		36.4	31.8	31.8	
Death												
Yes	15.8	36.8	47.4	0.146	16.7	40.0	43.3	0.070	25.0	37.5	37.5	0.450
No	40.0	31.1	28.9		40.6	29.7	29.7		36.2	31.9	31.9	

[a]P-value when stages I/II and III/IV were grouped. VI, vascular invasion; LNM, lymph node metastases; TD, tumor differentiation.

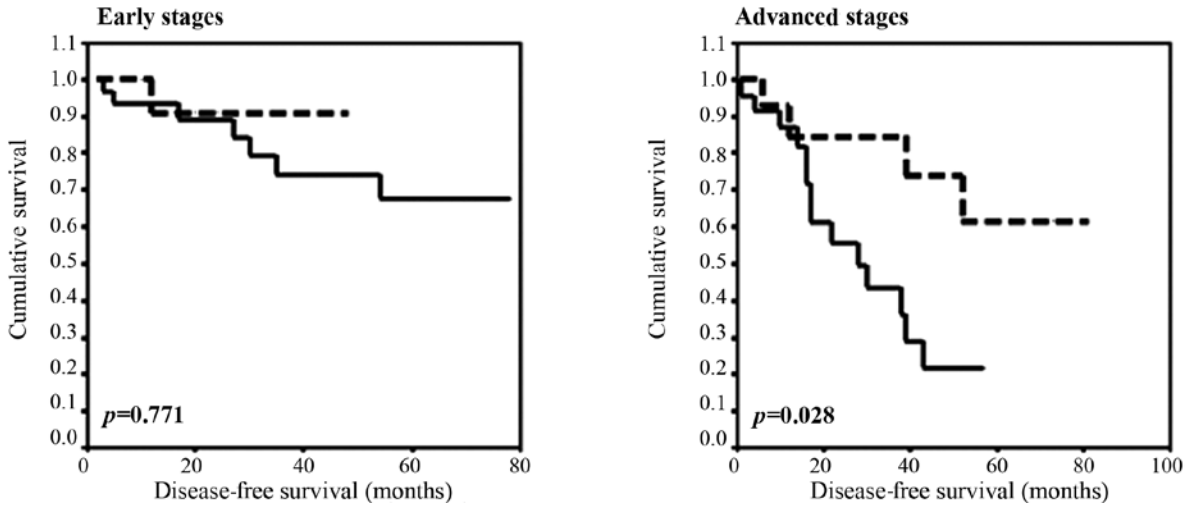

Figure 1. Kaplan-Meier disease-free survival curves in relation to the levels of *EPAS1* mRNA in plasma from CRC patients. Continuous line, high mRNA levels; dotted line, low mRNA levels.

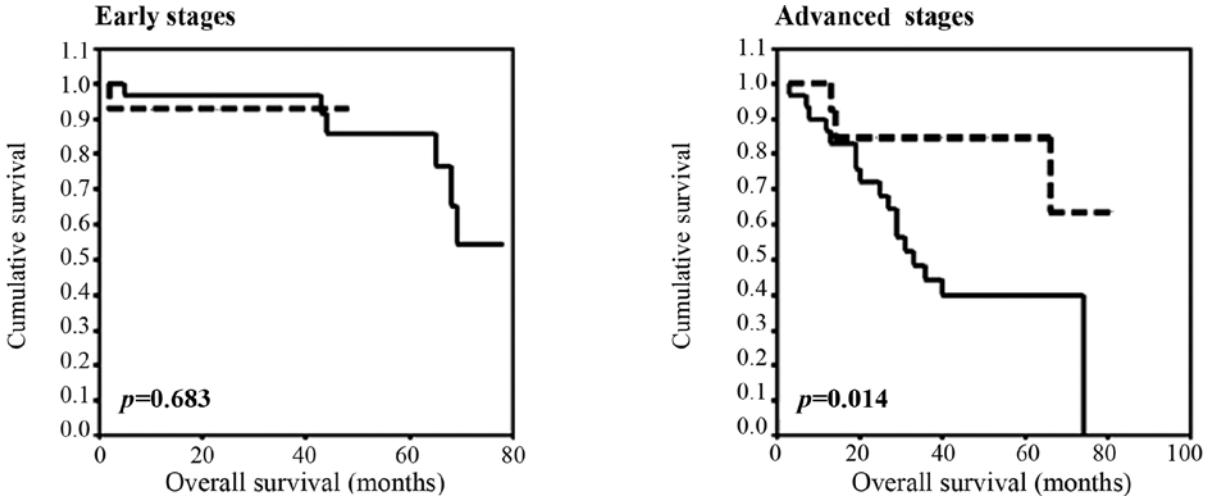

Figure 2. Kaplan-Meier overall survival curves in relation to the levels of *EPAS1* mRNA in plasma from CRC patients. Continuous line, high mRNA levels; dotted line, low mRNA levels.

CRC patients. This large series was independent of the series analyzed in our previous study (12). The presence of mRNA in plasma was identified in 64% of patients for *EPAS1*, in 90% for *UBE2D3* and in 44% for *KIAA0101*.

To evaluate the prognostic value of these markers, their possible association with clinicopathological parameters of the tumors indicative of poor prognosis was analyzed. A total of 43% of the patients were females and 57% were males, with 95% of the patients at >50 years of age. Vascular or lymphatic invasion was found in 28% of the patients. A total of 32% of the specimens were classified as well-differentiated, 57% as moderately differentiated and 11% as poorly differentiated. The majority of tumors were at intermediate stages: 11% of cases were classified as TNM I, 35% were TNM II, 39% were TNM III and 15% were TNM IV. For this purpose, the plasma mRNA levels of each gene were divided into tertiles (low, medium and high levels). Statistically significant associations were not found between mRNA levels of *KIAA0101* or *UBE2D3* and clinicopathological parameters. In the case of *EPAS1*, high levels of mRNA in the plasma correlated statistically with age (≥50 years) and relapse (p=0.012 and p=0.045, respectively; Pearson's Chi-square test). Moreover, we found a trend towards statistical association between high expression levels of *EPAS1* and patient mortality (p=0.07). No statistically significant correlations were noted between *EPAS1* levels and the remaining pathological parameters analyzed. The results are summarized in Table II.

Patients with medium or high levels of *EPAS1* mRNA in the plasma were found to have a distribution of data that was similar to patients with low *EPAS1* levels (Table II). Thus, patients exhibiting medium and high *EPAS1* levels were grouped together, and only two categories were analyzed (low and high). Statistical significance was evident between high levels of *EPAS1* with the clinicopathological parameters of age and relapse (p=0.003 and p=0.013, respectively; Pearson's Chi-square test). Furthermore, a statistical association was found between high levels of *EPAS1* mRNA and patient mortality (p=0.021).

Association of mRNA levels with survival of CRC patients. Patients were followed up for an average of 33.5 months (range 1-90). During this period, of the 154 CRC patients examined in this study, 33.3% had relapsed at the time of the last follow-up and 30% had succumbed to the disease. At 48 months follow-up, DFS was 52.9% (95% CI 42.2-63.6) and OS was 64.9% (95% CI 55.1-74.7).

Disease-free survival. No apparent differences were observed regarding *KIAA0101*, *EPAS1* and *UBE2D3* mRNA levels in the plasma and DFS following the Kaplan-Meier analysis. However, when patients divided into tertiles on the basis of their *EPAS1* mRNA levels were sub-grouped as early (I + II) and advanced (III) stages, a trend towards statistical association in the advanced stages (III) was observed (p=0.082, Kaplan-Meier test). As mentioned above, patients with medium and high levels of *EPAS1* mRNA levels in the plasma were grouped together and only two categories were analyzed: patients with low or high *EPAS1* levels. Therefore, statistical significance was evident for the mRNA levels of *EPAS1* in advanced stages of disease; patients with high levels of mRNA showed a 4-year DFS rate of 21.7% (95% CI 0.9-42.5), while patients with low levels showed a rate of 73.9% (95% CI 47.8-99.9) (p=0.028, Kaplan-Meier test, Fig. 1).

Overall survival. No differences were observed in OS for *KIAA0101* and *UBE2D3* mRNA levels in the plasma. However, a statistical association was found between high levels of *EPAS1* mRNA and shorter OS in patients at advanced stages (III + IV) (p=0.047, Kaplan-Meier test). As with DFS, patients with medium and high levels of *EPAS1* mRNA levels in the plasma were grouped together. The statistical significance was evident for the high mRNA levels of *EPAS1* at advanced stages of disease. Thus, at 48 months follow-up for the advanced stage patients, OS for patients with high levels was 39.8% (95% CI 20.8-58.9), while that of patients with low levels was 84.6% (95% CI 65-100) (p=0.014, Kaplan-Meier test, Fig. 2).

Discussion

Since genetic alterations are associated with an altered expression of numerous genes at the mRNA level, plasma RNA is a useful source of molecular information associated with tumor development. The presence of RNA released from tumor cells in the plasma of cancer patients has been proven. Plasma RNA in cancer patients circulates highly protected by tumor-specific microvesicle-like structures (18). Moreover, these released microvesicles efficiently transfer content to other cell types and have various effects on immune responses, cell growth, angiogenesis and metastasis (19-21). Plasma RNAs may provide prognostic information similar to RNA from primary tumors. The potential use of plasma RNA in this field has been reported (2,7,11,22,23). In this sense, the application of genomic profiling of plasma RNA may allow the unbiased selection of a cancer marker in the plasma (19). In the context of cancer research, DNA microarrays have been widely used to identify changes that are common to various types of cancer, as well as signature profiles unique to a sub-category of cancer. Furthermore, this technology may be useful in the detection of early stages of the disease, aiding prognosis, predicting response to specific therapies and monitoring the treatment of disease (24).

In a previous study, the feasibility of a genomic approach to studying plasma RNA was analyzed (12), as well as its diagnostic value. A set of four genes (*EPAS1*, *KIAA0101*, *UBE2D3* and *DDX46*) was selected from a list of 40 differentially expressed genes identified after profiling plasma RNA from CRC and from healthy donors by cDNA microarray hybridization. The four genes were analyzed by quantitative RT-PCR in new plasma samples, which confirmed higher mRNA levels of *EPAS1*, *KIAA0101* and *UBE2D3* in the plasma of CRC patients as compared to normal subjects. The levels of *EPAS1* and *UBE2D3* mRNA were significantly lower in plasma samples obtained after surgery, returning to normal levels, which shows a favorable correlation between these indicators and the tumor condition. Moreover, class prediction using support vector machines with a training set composed of real-time PCR data for *EPAS1* classified pre-surgery samples as the CRC group and post-surgery samples as the normal group (12).

In the present study, the mRNA level of *EPAS1*, *KIAA0101* and *UBE2D3* in plasma was analyzed to evaluate the prognostic value of the four genes. Although the mRNA levels of *KIAA0101* and *UBE2D3* in plasma may have diagnostic value (12), we found no prognostic value of these indicators due to associations with clinicopathological parameters and patient survival. However, high mRNA levels of *EPAS1* in plasma were associated with relapse of disease and patient mortality, as well as with shorter DFS and OS in advanced stages. *EPAS1* is a factor induced under hypoxia and is an essential mediator of cell adaptation to hypoxia, regulating the expression of genes involved in tumor angiogenesis, glucose metabolism and resistance to oxidative stress (25-27). The overexpression of *EPAS1* has been characterized in the tumor tissue of various types of human cancer, including CRC, and has shown a close correlation with metastatic tumor activity (28-30). However, previous data on *EPAS1* and clinical outcome in CRC have been inconclusive, showing both associations and no associations with poor survival (30-32). Our results indicate that the results reported by other investigators, who have shown that *EPAS1* is an indicator of poor prognosis in tumors, a reasonable assumption given the roles of this gene, may also be reflected in the plasma of patients in advanced stages.

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