THE EGYPTIAN JOURNAL OF IMMUNOLOGY Vol. 17 (2), 2010
Page: 121-129

Hypoxia-Induced EMAP-II Transcription in Colorectal Cancer

[1]Manal M. S. Youssef, [2]Yee M. Heng, [3]Desmond G. Powe, [4]Jodie Edgson, [5]I. O. Ellis, [6]Clifford Murray

[1]Clinical Pathology Department, Faculty of Medicine, Minia University, Egypt, [2,6]Wolfson Digestive Diseases Centre, University Hospital, [3,4,5]Division of Pathology, School of Molecular Medical Sciences, University of Nottingham, Nottingham, United Kingdom.

Endothelial monocyte–activating polypeptide-II (p43/EMAP-II) is a proinflammatory cytokine and a chemoattractant for mononuclear phagocytes and polymorphonuclear leucocytes, found in culture supernatants of many tumour cell lines. It was demonstrated that p43/EMAP-II induces apoptosis in mitogen-stimulated lymphocytes, and suggested that it may be a constituent of a novel immune evasion mechanism employed by tumour cells. Quantitative real-time reverse transcription- polymerase chain reaction (qRT-PCR) analysis for EMAP-II mRNA was performed for colorectal adenocarcinoma cell lines, DLD-1, HT 29; human umbilical vein endothelial cells (HUVEC); and normal colon under normal and hypoxic conditions. Under hypoxic conditions, EMAP-II transcript expression increased up to 22-fold over normoxia in tumour cells, while there was 1-fold increase due to hypoxia in HUVEC and no increase in normal colon. These results demonstrate that EMAP-II transcripts are upregulated in tumour cells in hypoxic conditions and support the notion that EMAP-II plays a complex and important role in human cancer.

Endothelial-monocyte-activating polypeptide-II (EMAP-II) was first detected in supernatants of cultured murine tumour cells (Berger *et al,* 2000; Murray and Tas, 2001) based on its ability to stimulate procoagulant activity in cultured endothelial cells. Kao *et al.* (1992) isolated 20–22 kDa EMAP-II protein, which proved to have pleiotropic, cytokine-like activity toward endothelial cells as well as monocytes and neutrophils (Kao *et al.,* 1994). It was later identified as an anti-angiogenic molecule, which induces apoptosis in proliferating and hypoxic endothelial cell in vitro and in angiogenic tumor vasculature *in vivo* (Schwarz *et al.,* 1999; Berger *et al.,* 2000). This apoptotic activity can be explained by the ability of EMAP II to activate the proapoptotic splice variant of the chemokine receptor CXCR3B (Hou *et al.,* 2006) and to compete with VEGF for binding to the VEGF receptor -2 (Awasthi *et al.,* 2009).

EMAP-II is synthesized as a 34-kDa precursor molecule, which is proteolytically cleaved to produce the 20-kDa mature polypeptide (Tas *et al,* 1997). EMAP-II and the p43 auxiliary component of the mammalian multisynthetase complex share a high degree of amino acid identity (Quevillon *et al,* 1997). Murray *et al* (2004) showed that EMAP-II might act in an immune-suppressive role, simultaneously triggering apoptosis in activated lymphocytes.

Tumour hypoxia is an important indicator of cancer prognosis; it is associated with aggressive growth, metastasis, and poor response to treatment (Höckel *et al,* 1996; Brizel *et al,* 1996). Of potential importance for understanding these effects is the role of hypoxia in regulating patterns of gene expression (Price *et al,* 1996; Heacock *et al,* 1986; Shweiki *et al,* 1992; Koong *et al,* 2000). Previous studies have reported hypoxia as a potent inducer of EMAP II release *in vitro* (Barnett *et al,* 2000). It was suggested that

apoptosis and hypoxia employ two distinct mechanisms leading to the release of EMAP (Matschurat *et al,* 2003).

In the past 10 years, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) has become one of the techniques of choice to study gene expression in cells and tissues. qRT-PCR assays have been used for quantitative determination of gene expression levels (Ginzinger, 2002; de Kok *et al.,* 2000; Homey *et al.,* 2000; Kubo *et al.,* 2000). In this study, qRT-PCR technique using real time fluorescence reporter molecules was used to determine EMAP-II transcription patterns under normal and hypoxic conditions in colorectal cancer.

Materials and Methods

Cell lines

The colorectal adenocarcinoma cell lines DLD-1, HT29; normal colon cells; and human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (Manassas, VA).

Cell culture and induction of hypoxia *in vitro*

Colorectal cancer cell lines were cultured in RPMI-1640 medium (Gibco, UK) supplemented with 10% fetal calf serum (PAA Laboratories, Lintz, Austria) and 100 U/ml penicillin/streptomycin solution (Sigma-Aldrich, Poole, UK). HUVEC were grown to confluence on 0.2% gelatin-coated tissue culture flasks in HUVEC medium M199 medium (Gibco Br, Paisley, UK) supplemented with 14Mm L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 90 µg/ml heparin, 20 µg/ml endothelial cell growth supplement (EGCS) (Boehringer Mannheim) and 20% fetal calf serum. Normal colon cells have been initiated and maintained in medium consisting of 45% Dulbecco's minimum essential medium and 45% Ham's F12 medium supplemented with antibiotics (100 U/ml penicillin/streptomycin solution); 0.005 mg/ml insulin (Sigma-Aldrich); 0.005 mg/ml transferring (Sigma-Aldrich); 100 ng/ml hydrocortisone (Sigma-Aldrich); fetal bovine serum (10%); and HEPES 25 mM (Sigma-Aldrich). Cells were maintained at 37°C in 5% CO_2 in a humidified incubator and were routinely subcultured by removal from flasks with 0.05% trypsin/1mM EDTA (Sigma-Aldrich).

For exposure to a hypoxic environment, subconfluent cells in serum-free medium were incubated in a hypoxic chamber containing 1% O_2, 5% CO_2, and 94% N_2 for 6 and 24 hours and in normal conditions as a control. Cell pellets were stored at –80 ^oC.

RNA extraction and cDNA synthesis

RNA from cultures of colorectal cancer cells was purified using a Purescript RNA Isolation Kit (Gentra Systems Inc., USA). To extract RNA, 1-2 million cells suspended in balanced salt solution were added to1.5 ml microfuge tubes on ice. 300 ul-cell lysis solution was added to the resuspended cells. 100 µl protein-DNA precipitation solution was added to the cell lysate. After incubation for 5 minutes on ice, the samples were centrifuged at 13,000-16,000 x g for 3 minutes. The supernatant containing the RNA was collected into a clean microfuge tube containing 300 µl 100% isopropanol. The samples were mixed and centrifuged at 13,000-16000 x g for 3 minutes. The supernatant was poured off and 300 µl 70% ethanol was added to wash the RNA pellet. The samples were centrifuged at 13,000-16,000 x g for 1 minute; air dried and rehydrated in 50 µl RNA hydaration solution for at least 30 minutes on ice. RNA samples were stored at -80^oC until use.

DNA was synthesized from total RNA using the enhanced Avian HS RT-PCR Kit (Sigma-Aldrich), in accordance with the manufacturer's protocol.

Real-time PCR of EMAP-II

qRT-PCR analysis for EMAP-II mRNA and 28S rRNA (endogenous RNA control) was performed using the Rotor-Gene 3000 (Corbett Research, Australia). 28S is recommended as internal standard for mRNA quantification (Thellin *et al,* 1999). The sequence of the primers used were as follows:
EMAP-II forward 5'-TAGCTGGAAGTGCCATGGAC TCTAAGCCAATAG -3', reverse 5'-AGCTCCTTGTC GACAGGCTCATCCTGGGAAAGC -3';
28S forward 5'-GGTGTCAGAAAAGTTACCACAG-3', reverse 5'- AATCCCACAGATGGTAGCTTCG-3'.

For qRT-PCR, The PCR DyNAmo HS SYBR Green (FINNZYMES, Finland) kit was used. Real-time PCR reactions were set up in triplicates for EMAP-II and housekeeping gene in each sample analysed. The PCR reaction contained 25 µl Master mix, 0.5M concentrations of each of the primers, 1 µl of the template cDNA, ddH_2O to make up to the final reaction volume 50 µl. The cycling conditions were as follows: 95°C for 10 minutes for 1 cycle, followed by 45 cycles of 95°C for 10 seconds, 64°C for 15 seconds, and 72°C for 20 seconds. A negative master mix contained water

instead of cDNA samples was used as a negative control. The primers were selected to work under identical PCR cycling conditions with an amplification efficiency that was approximately equal to 100%.

Fluorescence was measured following each cycle and displayed graphically (Real-time Detection System Software, version 6.0.19, Australia). The software determined a cycle threshold (C_T) value, which identified the first cycle at which the fluorescence was detected above the baseline for that sample.

Methodology of relative quantification of mRNA transcripts

The relative quantification of mRNA transcripts was carried out by the comparative C_T ($\Delta\Delta C_T$) method, the theoretic basis of which was previously described (Inoue *et al,* 2002; Ishida *et al,* 2000). For each experimental sample, the difference between EMAP-II C_T value and the C_T value of 28S was used to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step (ΔC_T). For relative quantification by the comparative C_T method, values are expressed relative to a sample called calibrator. The calibrator is the weakest signal from the normalization (ΔC_T) in each cell line. HUVEC cell line was chosen as a calibrator because it has the lowest expression level of EMAP-II. The ΔC_T for each experimental sample was subtracted from the ΔC_T of the calibrator ($\Delta\Delta C_T$). The amount of EMAP-II (linear value), normalized to an endogenous reference (28s RNA) and relative to the calibrator, was determined by evaluating the expression $2^{-\Delta\Delta C_T}$. C_T a numerical value given as parameters by Rotor Gene 3000 software (Corbett Research).

Statistical Analysis

The data were expressed as mean ± S.E. from three independent experiments and the statistical significance was tested by the student's *t*-test. A p value of <0.05 was considered statistically significant.

Results

Quantitative RT-PCR using EMAP-II primers

The standard curves for EMAP-II and 28S rRNA transcripts were linear (Figure 1A). The amplification efficiency of EMAP-II was equal to that of the reference (28S), which proved the validity of using the $\Delta\Delta C_T$ calculation for the relative quantification of EMAP-II. Three genes (28S, GADPH, and B-actin) were tested for their use as reference gene for Q-RT-PCR studies. 28S showed the least variability in expression among the cell lines (supplementary figure 1); therefore, it was selected as the reference gene. Melting-curves analyses indicated that the PCRs produced a single specific product (Figure 1B). Furthermore, melting-curves analysis showed that the samples did not produce any peaks indicating the absence of non-specific products (dimmers). Plots of fluorescence versus PCR were generated by real-time detection System Software (Figure 1C, D). The cycle threshold (C_T) for a fluorescent PCR correlates with amount of initial template in the PCR.

Influence of hypoxia on the quantification of EMAP-II mRNA

EMAP-II transcription in the various cell lines was assessed at 6 and 24 hours in normal and hypoxic conditions. We extrapolated the relative quantification of EMAP-II transcripts with the comparative C_T ($\Delta\Delta C_T$) method using 28S as an endogenous reference. EMAP-II transcripts reached highest levels in hypoxic colorectal cancer cell lines compared with normal control (Table 1).

Transcription of EMAP-II in hypoxic DLD-1 cell line was 12-21-fold higher than in normal conditions ($P<0.05$) (Figure 2A). Levels of EMAP-II in hypoxic HT29 cell line were 10-15-fold higher than in normal cells ($p<0.05$) (Figure 2B). In contrast to CRC, EMAP-II expression levels in HUVEC did not increase significantly in hypoxia (Figure 2C). In normal colon, levels of EMAP-II transcriptions remained nearly the same in normal and hypoxic conditions (Figure 2D). Levels of EMAP-II rose over time in hypoxia, while no significant transcription of EMAP-II was detected over time in any other cell (Figure 2).

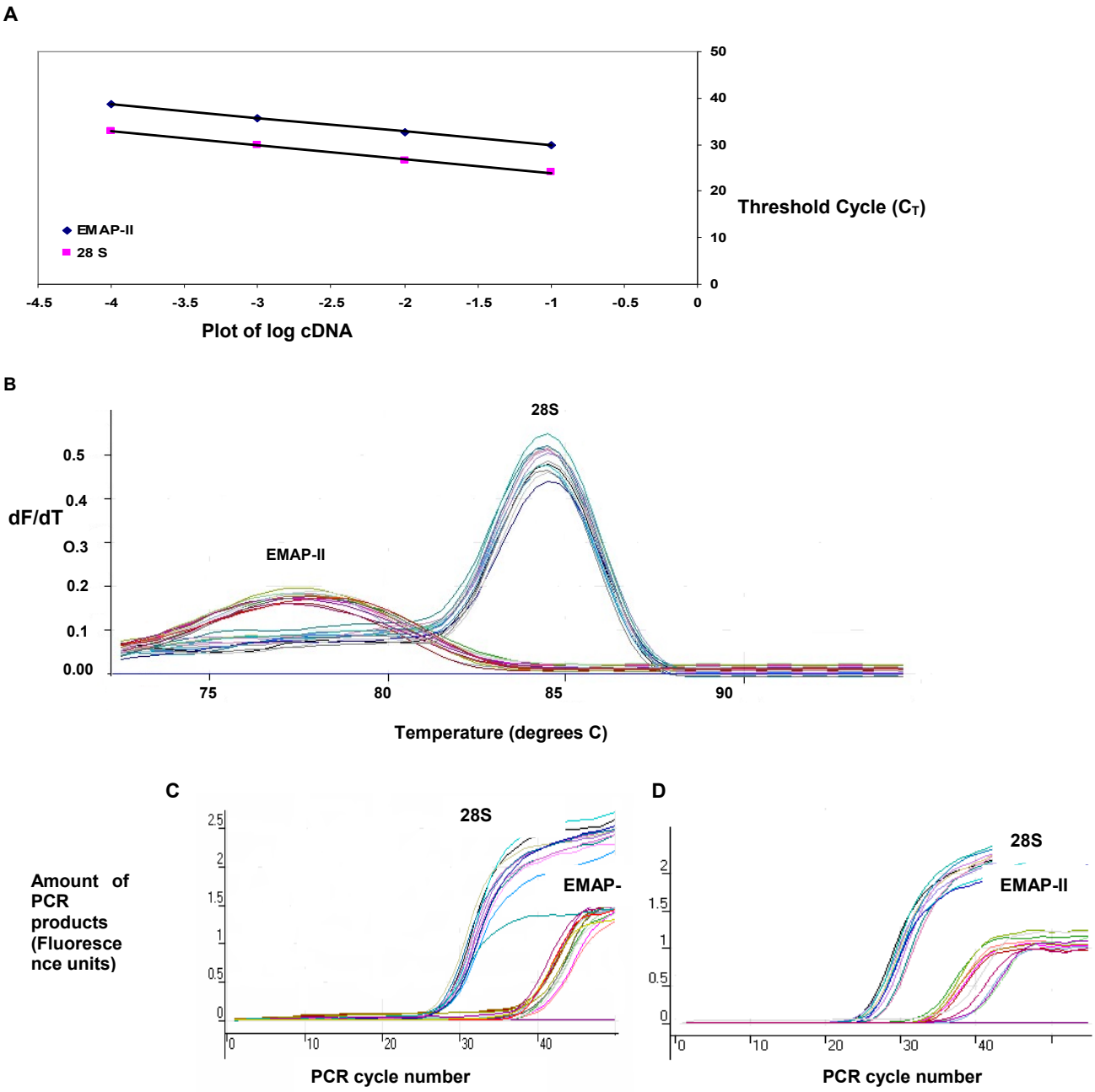

Figure 1. The RT-PCR of EMAP-II transcripts using EMAP-II primers in colorectal cancer. (A) Real-time RT-PCR standard curves. The curves are generated by plotting the log of the starting quantity cDNA versus the C_T value. Three replicates were performed for each standard curve. EMAP-II, y = -2.92x + 26.95, r = 0.9986; 28S, y = -2.98x + 20.9, r = 0.9976. The efficiencies of target and reference are approximately equal. (B) A representative melting-curve analysis of PCR products. The fluorescence versus temperature for EMAP-II and 28S PCR products. Each trace is the derivative of the fluorescence versus temperature (dF/dT) plot, which indicates where the plot changes slope and the temperature at which the duplex melt. There was a single peak for all melting-curve traces, which suggests a single size product that is assumed to be the specific PCR product. (C) Amplification plot of real time qRT-PCR of DLD-1 samples normalised with 28S under normal and hypoxic conditions. The y-axis represents the RFU (relative fluorescence units). Cycle number is displayed on the x-axis. (D) Amplification plot of real time qRT-PCR of HT29 samples normalised with 28S under normal and hypoxic conditions. The y-axis represents the RFU (relative fluorescence units). Cycle number is displayed on the x-axis.

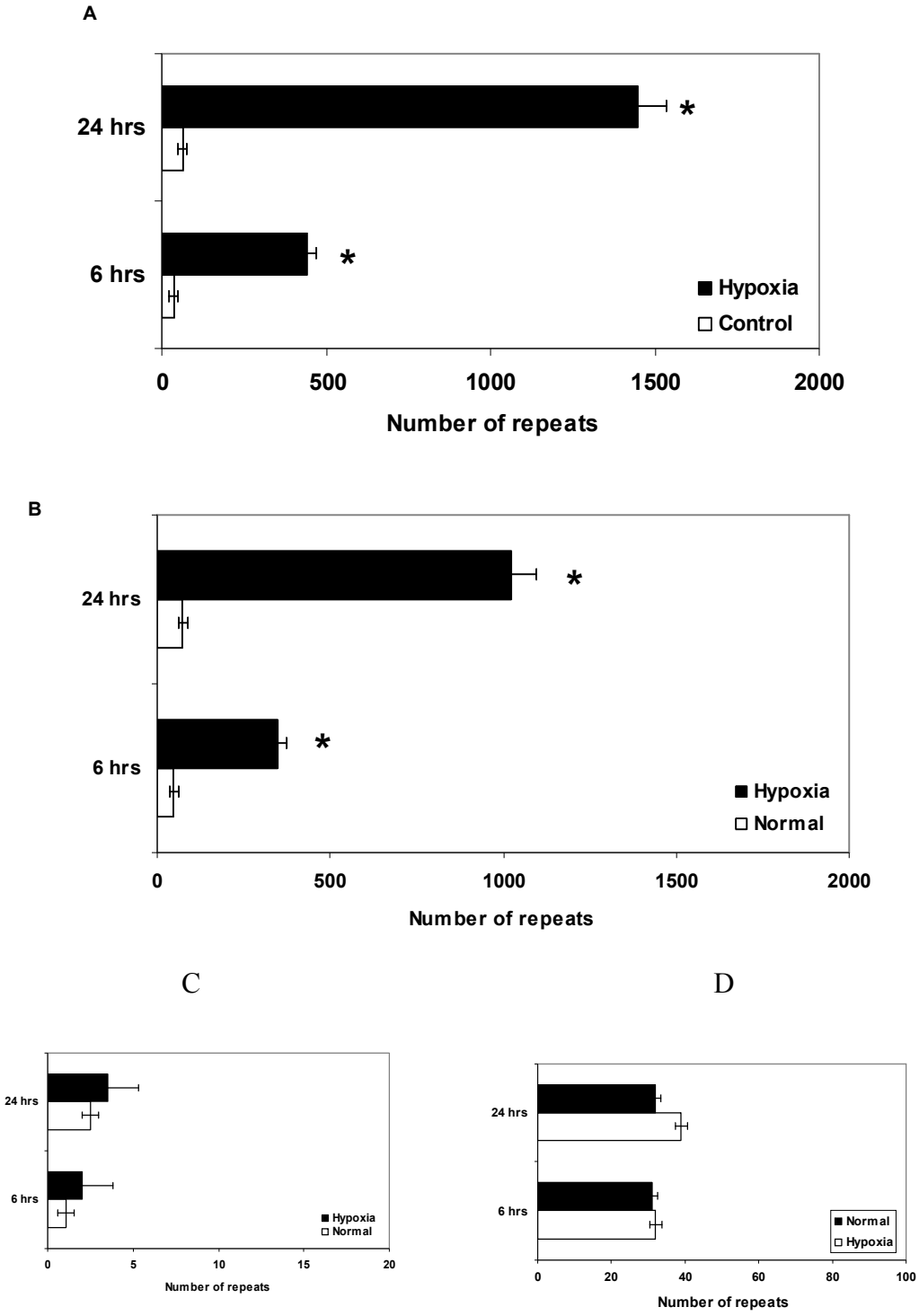

Figure 2. Effect of hypoxia on EMAP-II level determined using real-time RT-PCR. A significant difference was seen in EMAP-II expression between normal and hypoxic DLD-1 (A), and HT29 (B) cells. There was no significance between normal and hypoxic normal colon cell (C), and HUVEC (D). The data shown are the averages of three experiments (mean ± SEM). ** Indicates significance at $P<0.05$ in comparison to controls.

Table 1. Relative quantification of target EMAP-II using the comparative ($\Delta\Delta C_T$) method. P<0.05 by the test. The ΔC_T was determined by subtracting the average reference (28S) C_T value from the average EMAP-II ΔC_T value. The calculation of $\Delta\Delta C_T$ involved subtraction by the ΔC_T value of the calibrator

Target/ reference	Samples	DLD-1		HT29		HUVECS		Normal colon	
		ΔC_T±SD	$2^{-\Delta\Delta C}{}_T$	ΔC_T±SD	$2^{-\Delta\Delta C}{}_T$	ΔC_T±SD	$2^{-\Delta\Delta C}{}_T$	ΔC_T±SD	$2^{-\Delta\Delta C}{}_T$
EMAP-II/28S	6 Normal	10.81	37	10.44	47.1	16	1.07	11	32
	6 Hypoxic	7.19	445*	7.54	349*	15	2	11.22	27.4
	24 Normal	10	64	9.8	73	14.70	2.5	10.50	45
	24 Hypoxic	5.50	1448*	6	1024*	14.8	3.5	11.33	25.4

EMAP-II expression of colorectal cancer compared with normal tissue

EMAP-II mRNA levels in hypoxic colorectal cancer cells were significantly higher than that in normal colon (p<0.05) and HUVEC (P<0.05). On the other hand, there was no significant difference of EMAP-II mRNA levels between normal and hypoxic HUVEC or normal colon cells (Figure 3).

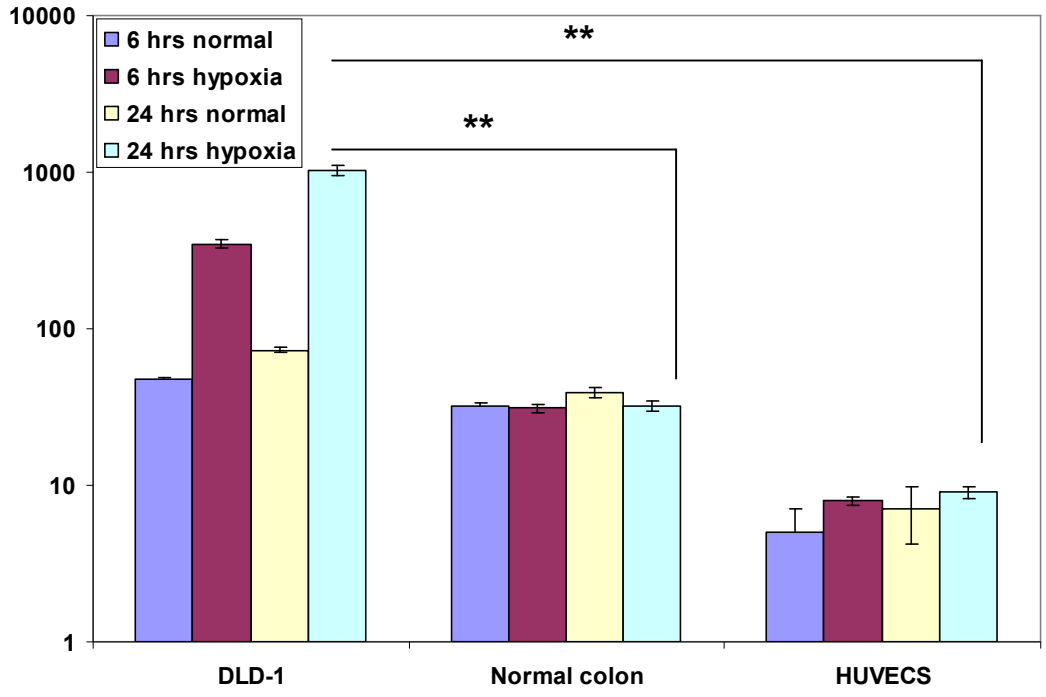

Figure 3. Comparison of EMAP-II mRNA expression between colorectal cancer DLD-1 and normal colon and HUVEC. The data shown are the averages of three experiments (mean ± SEM). ** Indicates significance at p<0.05 in comparison to controls.

Discussion

qRT-PCR using EMAP-II primers was used to rapidly and reliably quantify the relative level of expression of EMAP-II during normal and hypoxic conditions. The PCRs using EMAP-II primers yielded data that were applied to the comparative C_T method for calculating the change in the relative expression between samples. We show an increase in EMAP-II transcripts in hypoxic cancer cells. Furthermore, we provide relative quantification of the increase in expression.

The goal of this study was to investigate EMAP-II-gene transcription levels using quantitative real-time PCR, and to determine the relationship between EMAP-II transcription and hypoxia in colorectal cancer. Real-time PCR (Heid *et al*, 1996; Kipar *et al,* 2001) allowed the analysis of EMAP-II transcription in a time dependent manner.

Colorectal cancer is characterized by regions of variable hypoxia (Moulder and Rockwell, 1984). Cells in these hypoxic regions are resistant to radiation and to cytotoxic drugs (Bush *et al,* 1978). Cells undergo numerous changes in gene transcription, enzyme activities in response to hypoxia (Li and Jackson, 2002). In this article, it was shown that EMAP transcript expression increased 8-22-fold over normoxia in tumour cells, while there was no change due to hypoxia in HUVEC or normal colon.

Transcripts encoding EMAP-II have been found in a wide range of human tissues as well as normal and tumour cell lines (Tas *et al,* 1997; Knies *et al,* 1998). To date, however, there is no information on the transcription profile of EMAP-II gene in normal tissues or tumours.

In the present study, hypoxic colorectal cancer cells were shown to express high levels of EMAP-II, suggesting that the hypoxic induction of EMAP-II may contribute to the resistance of hypoxic tumour cells to treatment. This observation contrasts with a previous with a previous study (Matschurat *et al.,* 2003) showing that. Hypoxic induction of EMAP-II mRNA detected by RT-PCR analyses of colorectal cancer may be due, at least, to transcriptional up-regulation. This finding is based on results from two cell lines. The same results were obtained in other two colorectal cancer cell lines (data not shown)

Previous studies showed that low oxygen levels can enhance EMAP-II release and conversion (Barnett *et al,* 2000). Hypoxia is known to up-regulate the expression and release of matrix metalloproteinases and plasminogen activator-1 from tumour cells (Canning *et al,* 2001; Koong *et al.,* 2000), and these enzymes could potentially be involved in EMAP-II expression. Matschurat *et al* (2003) showed that strongly proEMAP II/p43 expressing cells are located to areas of tissue hypoxia.

EMAP-II mRNA showed a significant correlation with the time in hypoxia for cancer cells. These results are consistent with a recent report of high levels of EMAP-II with increasing time (Youssef *et al,* submitted for publication). These results suggest the stability of EMAP-II gene. However, no significant levels of EMAP-II transcription were detected over time in normal colon or HUVEC.

However, the status differed for EMAP-II mRNA in normal colon, which showed no change in the relative quantification in hypoxic samples, thus suggesting the stability of EMAP-II in terms of hypoxia compared with cancer cells.

A potentially important finding in this study was that EMAP-II mRNA expression in hypoxic tumour cells was significantly higher than in both normal colon and HUVEC cells. If patients with colorectal cancer can be distinguished from those with normal colon

by measuring EMAP-II expression, it may result in a better strategy for treatment of colorectal cancer.

In summary, this study shows that mRNA levels for EMAP-II are increased in colorectal cancer during hypoxia and support the notion that EMAP-II has an important role in tumours. These studies indicate that increased expression of EMAP-II in tumours could have important implications for tumour growth, spread, and response to treatment.

Acknowledgment

We would like to express special thanks to Cancer Research, United Kingdom for providing us with supports for this work.

References

1. Awasthi N, Schwarz MA, Verma V, Cappiello C, Schwarz RE (2009). Endothelial monocyte activating polypeptide II interferes with VEGF-induced proangiogenic signaling. Lab Invest 89:38–46.
2. Barnett G, Jakobsen AM, Tas M, Rice K, Carmichael J, Murray JC (2000). Prostate adenocarcinoma cells release the novel proinflammatory polypeptide EMAP-II in response to stress. Cancer Res 60: 2850-57
3. Berger AC, Tang G, Alexander HR, Libutti SK (2000). Endothelial monocyte-activating polypeptide-II, a tumour-derived cytokine that plays an important role in inflammation, apoptosis, and angiogenesis. J Immunother 23: 519-27
4. Brizel DM, Scully SP, Harrelson JM, et al. (1996). Tumour oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 56: 941–43
5. de Kok JB, van Balken MR, Roelofs Rwhm, van Aarssen Yawg, Swinkels DW and Gunnewiek Jmtk (2000). Quantification of hTERT mRNA and telomerase activity in bladder washings of patients with recurrent urothelial cell carcinomas. Clin *C*hem 46: 2003-07
6. Ginzinger DG (2002). Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 30: 503-12
7. Heacock CS, and Sutherland RM (1986). Induction characteristics of oxygen regulated proteins. Int J Radiat Oncol Biol Phys 12: 1287–90
8. Höckel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996). Association between tumour hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1: 4509-15
9. Homey B, Dieu-Nosjean MC, Wiesenborn A, et al (2000). Up-regulation of macrophage inflammatory protein-3 alpha/ CCL20 and CC chemokine receptor 6 in psoriasis. J Immunol 164: 6621-32
10. Hou Y, Plett PA, Ingram DA, Rajashekhar G, Orschell CM, Yoder MC, March KL, Clauss M. (2006). Endothelial-monocyte-activating polypeptide II induces migration of endothelial progenitor cells via the chemokine receptor CXCR3. Exp Hematol 34:1125–32.
11. Inoue H, Kimura A, Tuji T (2002). Degradation profile of mRNA in a dead rat body: basic semi-quantitation study. Forensic Sci Int 130: 127-32
12. Ishida K, Zhu BL, Maeda H (2000). Novel approach to quantitative reverse transcription PCR assay of mRNA component in autopsy materials using the taqman fluorogenic detection system: dynamics of pulmonary surfactant apoprotein A. Forensic Sci Int 113: 127-31
13. Kao J, Ryan J, Brett J, Chen J, Shen H, Fan YG, Godman G, Familletti PC, Wang F, Pan YC (1992). Endothelial monocyte-activating polypeptide-II. A novel tumour-derived polypeptide that activates host-response mechanisms. J Biol Chem 267: 20239-47
14. Kao J, Houck K, Fan Y, Haehnel I, Libutti SK, Kayton ML, Grikscheit T, Chabot J, Nowygrod R, Greenberg S (1994). Characterization of a novel tumour-derived cytokine. Endothelial monocyte-activating polypeptide II. J Biol Chem 269: 25106-19
15. Koong AC, Denko NC, Hudson KM, et al. (2000). Candidate genes for the hypoxic tumour phenotype. Cancer Res 60: 883- 7
16. Matschurat S, Knies UE, Veronika Person, et al. (2003). Regulation of EMAP-II by Hypoxia. Am J Pathol 162: 93–103
17. Kubo A, Nishitani V, Minamino N, et al (2000). Adrenomedullin gene transcription is decreased in peripheral blood mononuclear cells of patients with IgA nephropathy. Nephron 85: 201-6

18. Murray JC, Tas MPR (2001). Endothelial monocyte-activating polypeptide 2: a novel injury signal? In The New Angiotherapy (TP Fan and EC Kohn, Eds.), Clifton NJ, Humana p: 491-98

19. Murray JC, Symonds P, Ward W, Huggins M, Tiga A, Rice K, Heng YM, Todd I, Robins RA (2004). Colorectal cancer cells induce lymphocyte apoptosis by an endothelial monocyte-activating polypeptide-II-dependent mechanism. J Immunol 172: 274–81

20. Price BD, and Calderwood SK (1992). Gadd45 and Gadd153 messenger RNA levels are increased during hypoxia and after exposure of cells to agents, which elevate the levels of the glucose-regulated proteins. Cancer Res 52*:* 3814 -17

21. Quevillon S, Agou F, Robinson JC, Mirande M (1997). The p43 component of the mammalian multi-synthetase complex is likely to be the precursor of the endothelial monocyte-activating polypeptide-II cytokine. J Biol Chem 272: 32573-79

22. Schwarz MA, Kandel J, Brett J, Li J, Hayward J, Schwarz RE, Chappey O, Wautier JL, Chabot J, Lo Gerfo P, Stern D. (1999). Endothelial-monocyte activating polypeptide II, a novel antitumor cytokine that suppresses primary and metastatic tumor growth and induces apoptosis in growing endothelial cells. J Exp Med 190:341–54

23. Shweiki D, Itin A, Soffer D, and Keshet E (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (Lond.) 359: 843-5

24. Tas MPR, Houghton J, Jakobsen AM, Tolmachova T, Carmichael J, Murray JC (1997). Cloning and expression of human endothelial-monocyte-activating polypeptide 2 (EMAP-2) and identification of its putative precursor. Cytokine 9: 535-53

25. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999). Housekeeping genes as internal standards: use and limits. J Biotech 75: 291-5.