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HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients

Miroslav Svoboda[1,2,*,†], Jana Slyskova[1,2,†], Michaela Schneiderova[3], Peter Makovicky[4], Ludovit Bielik[1,2,5], Miroslav Levy[6], Ludmila Lipska[6], Beata Hemmelova[7], Zdenek Kala[7], Marketa Protivankova[8], Ondrej Vycital[9,10], Vaclav Liska[9,10], Lucie Schwarzova[2], Ludmila Vodickova[1,2] and Pavel Vodicka[1,2]

[1]Institute of Experimental Medicine, ASCR, Prague 14220, Czech Republic, [2]Institute of Biology and Medical Genetics, First Medical Faculty, Charles University, Prague 12000, Czech Republic, [3]Department of Surgery, General University Hospital in Prague, First Medical Faculty, Charles University, Prague 12111, Czech Republic, [4]Department of Veterinary Sciences, Czech University of Life Sciences, Prague 16521, Czech Republic, [5]Faculty of Science, Charles University, Prague 12800, Czech Republic, [6]Department of Surgery, Thomayer Hospital and First Faculty of Medicine, Prague 14200, Czech Republic, [7]Department of Surgery and [8]Department of Internal Medicine, Haematology and Oncology, University Hospital Brno and Masaryk University, Brno 62500, Czech Republic, [9]Department of Surgery, Medical School and Teaching Hospital Plzen, Charles University, Plzen 30606, Czech Republic and [10]Biomedical Centre, Medical School Plzen, Charles University in Prague, Plzen 30605, Czech Republic

*To whom correspondence should be addressed. Tel: +420 296 442 251; Fax: +420 296 442 782;
Email: svoboda@oncobios.org

Correspondence may also be addressed to Pavel Vodicka. Tel: +420 296 442 694; Fax: +420 296 442 782;
Email: pvodicka@biomed.cas.cz

Colorectal cancer (CRC) is one of the main causes of death of neoplasia. Demand for predictive and prognostic markers to reverse this trend is increasing. Long non-coding RNA HOTAIR (Homeobox Transcript Antisense Intergenic RNA) overexpression in tumors was previously associated with poor prognosis and higher mortality in different carcinomas. We analyzed HOTAIR expression levels in tumor and blood of incident sporadic CRC patients in relation to their overall survival with the aim to evaluate surrogate prognostic marker for CRC. Tissue donor group consisted of 73 CRC patients sampled for tumor and normal tissue. Blood donor group was represented by 84 CRC patients compared with 40 healthy controls. Patients were characterized for tumor-node-metastasis stage, tumor grade, microsatellite instability and tumor penetration by stromal cells. HOTAIR levels were assessed by real-time quantitative PCR. CRC patients had higher HOTAIR expression in blood than healthy controls ($P = 0.0001$), whereas there was no difference in HOTAIR levels between tumor and adjacent mucosa of CRC patients. HOTAIR levels positively correlated between blood and tumor ($R = 0.43$, $P = 0.03$). High HOTAIR levels in tumors were associated with higher mortality of patients [Cox's proportional hazard, hazard ratio = 4.4, 95% confidence interval: 1.0–19.2, $P = 0.046$]. The hazard ratio was even higher when blood HOTAIR levels were taken into account (hazard ratio = 5.9, 95% confidence interval: 1.3–26.1, $P = 0.019$). Upregulated HOTAIR relative expression in primary tumors and in blood of CRC patients is associated with unfavorable prognosis. Our data suggest that HOTAIR blood levels may serve as potential surrogate prognostic marker in sporadic CRC.

Abbreviations: cDNA, complementary DNA; CRC, colorectal cancer; HOTAIR, **H**omeobox **T**ranscript **A**ntisense **I**ntergenic **R**NA; lncRNA, long non-coding RNA; ncRNA, non-coding RNA; PBMC, peripheral blood mononuclear cells; qPCR, quantitative PCR; ROC, receiver operating characteristic; TNM, tumor-node-metastasis.

[†]These authors contributed equally to this work.

Introduction

Most of human genome is transcribed to RNA, but only ~2% of RNA encodes functional proteins (1,2). RNAs that lack protein-coding ability and do not contain open-reading frames longer than 100 nucleotides are referred to as non-coding RNAs (ncRNAs). NcRNAs are arbitrarily subdivided into groups of small ncRNAs and long ncRNAs (lncRNAs), the latter being longer than 200 nucleotides. lncRNAs are transcribed from at least 10 000 genomic sites located throughout all chromosomes (3). Biological roles of most lncRNAs are unknown or poorly understood. However, lncRNAs are recently increasingly emerging as molecules that take its part in human carcinogenesis. Although their exact mechanism contributing to carcinogenesis is almost unknown at present, altered expression of several lncRNAs has recently been attributed to pathogenesis of some malignant neoplasia, including colorectal cancer (CRC) (4).

An lncRNA HOTAIR (**H**omeobox **T**ranscript **A**ntisense **I**ntergenic **R**NA) takes part in epigenetic regulation of gene transcription. HOTAIR interacts on its 5′ end with Polycomb repressive complex 2 to remodel chromatin and ensure silencing of HOX genes during embryonic development, whereas on 3′ end HOTAIR interacts with histone demethylase (5). It is thus an important epigenetic factor involved in the embryonic differentiation of tissues. In human association studies, HOTAIR has been recognized to be related with metastatic activity of primary cancer and with the patients' prognosis. HOTAIR levels were significantly elevated in gastrointestinal stromal tumors (6) and >1000-fold in metastatic breast cancer (7). Link between poor patients prognosis and higher expression of HOTAIR was reported for pancreatic (8) and hepatocellular cancers (9). One of the possible mechanisms underlying these observations was brought up by *in vitro* study (7). The authors found that HOTAIR-induced overexpression in epithelial cancer cells caused genome-wide retargeting of Polycomb repressive complex 2, resulting in altered histone methylation, gene expression and finally, cancer invasiveness. Moreover, a repression of several important tumor suppressor genes and an induction of certain prometastatic genes were identified to explain prometastatic effect of high HOTAIR expression (7).

In the present study, we analyzed HOTAIR lncRNA levels in tumors and in blood of sporadic CRC patients and in association with their overall survival. There is only one study currently available focused on HOTAIR in relation to sporadic CRC, which showed that HOTAIR overexpression is associated with higher risk of metastasis and poor prognosis of patients (10). Levels of HOTAIR have not yet been studied in peripheral blood of CRC patients. Therefore, our aim was to explore whether tumor levels of HOTAIR may predict sporadic CRC patient's survival and if so, whether blood levels of HOTAIR might represent a surrogate prognostic marker. Furthermore, since higher tumor stage [tumor-node-metastasis (TNM) 3 and 4], its particular localization (left colon), histology (high content of stromal cells in tumor) and microsatellite instability are hallmarks of worse prognosis of CRC patients, HOTAIR relative expression in tumor tissue and blood have been analyzed with respect to all these clinicopathological parameters in our study. Sporadic CRC is still a leading malignancy in Western countries, regarding both incidence and mortality, partially due to a late diagnosis and a lack of cost-effective predictive and prognostic markers (11). Reliable predictive and prognostic marker, analyzed in non-invasively obtained surrogate samples, may have vast clinical potential.

Materials and methods

Study subjects

Incident CRC cases were recruited at the time of the diagnosis among patients treated at surgical departments of Thomayer Hospital in Prague, General

University Hospital in Prague, University Hospital in Brno and Medical School and Teaching Hospital in Pilsen, all located in the Czech Republic, between 2008 and 2012. Only histologically confirmed new CRC cases that had not been previously diagnosed for cancer or inflammatory bowel disease were included into the study. Collected clinical data contained information about tumor localization, TNM stage according to American Joint Committee on Cancer (12), tumor grade and microsatellite instability status analyzed according the reference (13). To evaluate overall survival (from diagnosis until death or censoring event), patients were followed-up over a period of 35 months on average (with range 12–54 months).

Study involved two different groups of sporadic CRC patients. The first group (tissue donors) consisted of 73 patients who underwent surgical resection or probatory biopsy of the tumor, and in 52 of them adjacent healthy tissue was available as well. Patients did not undergo any adjuvant therapy prior to sampling. Regarding 14 patients with rectal cancer who underwent preoperative neoadjuvant chemoradiotherapy, the tissue specimens were collected as a part of probatory biopsy to determine precise pretreatment TNM stage. The second group (blood donors) involved 84 patients who were sampled for peripheral blood, taken at the time of diagnosis, i.e. before surgery and any therapy. In a subgroup of 26 patients paired tumor and healthy tissues and peripheral blood were available. To investigate whether HOTAIR lncRNA can be detected in plasma, 12 patients and 8 controls were studied.

Blood-measured HOTAIR levels in CRC patients were compared with 40 healthy controls, recruited from healthy volunteers residing retirement homes, blood bank donors and older healthy relatives of our collaborators and laboratory staff. Only subjects with no previous diagnosis of CRC or any malignant disease and without manifestation of any acute disease or injury at the time of blood collection were enrolled. Controls had not been exposed to any potentially harmful chemicals except for those common to everyday environmental sources and were sampled without any particular requirement for fasting prior to blood sampling. Structured questionnaire was received from all blood donor CRC patients and healthy controls. They reported their smoking habits, alcohol consumption, body mass index (calculated from weight and height) and presence of diabetes. All participants were of Caucasian origin. Participating subjects were properly informed about the aims of the research; they signed a written consent and approval for molecular genetic analysis, in accord with the Helsinki declaration. The Ethics Committees of all collaborating hospitals approved the design of the study.

Histological examination of tumor and mucosa samples

Tumor samples were taken from macroscopically distinguishable tumor central area far from the visible tumor edge and necrotic sites. Mucosa samples were taken 5–10 cm distant from the tumor. Samples were immersed in RNA-preserving solution RNALater according to the manufacturer's recommendations (Life Technologies, Carlsbad, CA) immediately after tissue removal. Tissues were evaluated for carcinoma/stromal cells ratio by objective morphometry using the NikonElipse E600 microscope and by NIS-Elements version 3.0 software (Nikon Instruments, Amsterdam, Netherlands). Ten microscopic fields per sample were randomly selected and measured for content of stromal cells. The average of 10 measurements per sample was determined. Samples were categorized to a 'high' stroma group when >50% of tumor content was formed by stromal cells, the rest was attributed to 'low' stroma category.

Isolation of peripheral blood mononuclear cells

Peripheral venous blood was drawn from each subject into EDTA-K2 tubes (Greiner Bio-one, Frickenhausen, Germany), gently shaken and kept at 4°C until the processing, no longer than 1 h since the sampling. Two ml of blood were layered over Ficcoll-Paque™ Plus (Sigma–Aldrich, St. Louis, MO) and separated according to the manufacturer's recommendations. Isolated peripheral blood mononuclear cells (PBMC) were immediately mixed with 1 ml of Trizol reagent and stored at −80°C for subsequent RNA isolation. To obtain plasma, the whole blood was centrifuged at 160*g* at room temperature. Plasma was stored at −80°C.

RNA isolation and cDNA synthesis

Total RNA from tumors, healthy mucosa and blood plasma was isolated by a MirVana isolation kit (Life Technologies). Total RNA from PBMC was isolated using TRIzol according to the manufacturer's procedure (Life Technologies) and kept at −80°C. RNA concentration and purity was measured on a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). The $OD_{260/280}$ ratios for all samples were between 1.8 and 2.0. RNA integrity was determined using Agilent 2100 Bioanalyzer, with RNA 6000 Nano Assay Chip (Agilent Technologies, Santa Clara, CA). The RNA integrity of all samples ranged between 6.0 and 10.0. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA by using a RevertAid™ Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers in a final volume of 40 µl, following the manufacturer's instructions. The cDNA was stored at −20°C.

Reverse transcriptase qPCR

Reverse transcriptase quantitative PCR (qPCR) was performed on a real-time 7500 PCR system (Life Technologies) using Precision™ 2x qPCR Mastermix and custom-designed real-time PCR assays with PerfectProbe™ (Primer Design Ltd, Southampton, UK). TaqMan-based fluorescence detection (fluorogenic 5′-nuclease) assay for routine real-time PCR analyses was used. The most suitable reference housekeeping gene from a panel of commonly known references ACTB, GAPDH, HPRT, 18S rRNA, PPIA and GUS was selected using both Genorm and Normfinder algorithms implemented in Genex software. For the normalization of tissue-obtained data, GUS (glucuronidase) was used as the best reference gene, whereas PPIA (cyclophilin A) was selected for normalization of blood-obtained data. The sequence of HOTAIR primer set is as follows: sense: 5′-ACATTCTGCCC TGATTTCCG-3′, antisense: 5′-CTTACCCC CACGGAGCAG-3′ detecting transcript sequence under Accession Number NR_003716.

The PCR reactions were performed in a volume of 30 µl, containing 30 ng of cDNA for each sample. The cycling program was set for initial hold at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 50°C for 32 s and 72°C for 15 s. Each run contained positive (interplate calibrator, 30 ng of human cDNA) and negative (no template) control. Results were analyzed using integrated System SDS 7500 Software version 1.3.1 (Life Technologies). The detection limit was established by calibration curve experiments. The calibration curve was linear until C_T 35. HOTAIR levels exceeding cutoff value of C_T 35 were considered negative. Sensitivity and specificity of HOTAIR detection were calculated by means of receiver operating characteristic (ROC) curve method using MedCalc software. Data are expressed as relative to median quantities using $\Delta\Delta C_T$ method. Negligible role of relative expression evaluation method on the precision of reverse transcriptase qPCR data was confirmed by comparison of ROC curves from three different evaluation methods. The difference between evaluation methods was represented by the use of three different reference values: (i) sample with the lowest expression, (ii) percentage of total expression and (iii) median expression of control group. Method used for data evaluation had no effect on the area under the ROC curves (Supplementary Material S1, available at *Carcinogenesis* Online).

Statistical analysis

HOTAIR relative expression data did not exhibit normal distribution according to the Shapiro–Wilk test (SH-W = 0.597, $P = 0.000$) and were therefore processed by non-parametric tests (Mann–Whitney *U*-test, Kruskal–Wallis and Spearman correlation test). Survival rates were calculated by Kaplan–Meier survival analysis and log-rank test. Hazard ratios were evaluated by means of Cox proportional hazards model univariate and multivariate analyses. Statistical analyses were performed using Statistica 9 (StatSoft, Tulsa, OK), Medcalc (MedCalc, Ostend, Belgium), GraphPad Prism 6 (GraphPad Software, La Jolla, CA) and IBM SPSS Statistics 18 (IBM, NY) softwares.

Results

Characteristics of study population

Clinicopathological characteristics of CRC patients who provided tissue samples (tissue donors) or blood samples (blood donors) are depicted in Table I. Median age of tissue donors was 67 years (range 38–85) and they consisted of 46 males and 27 females. Median age of blood donors was 67 years (range 32–88), and this group involved 58 males and 26 females. Healthy controls were of similar age distribution as CRC patients, with median of 66 years (range 32–85; $P = 0.301$) and consisted of 16 males and 24 females. The effect of possible confounding factors (age, sex, smoking, alcohol intake, body mass index and diabetes) was assessed by logistic regression. None of the confounders influenced HOTAIR expression neither in blood donors nor healthy controls (Supplementary Material S2, available at *Carcinogenesis* Online).

HOTAIR relative expression in tumor versus healthy mucosa

There was no difference in HOTAIR relative expression between tumor and adjacent cancer cells-free mucosa (1.18 versus 1.00, $P = 0.81$; Figure 1A), and expression levels correlated between both tissues ($R = 0.32$, $P = 0.022$). HOTAIR relative expressions in tumors were not significantly associated with any investigated clinicopathological features (Table I). The ratio of stromal cells content in the tumor had no influence on HOTAIR relative expression (1.11 versus 1.21, $P = 0.44$).

Table I. Clinicopathological characteristics of tissue donor and blood donor CRC patients and their respective HOTAIR relative expression levels measured in tumor and blood samples

		Tissue donors, *n* (%)	HOTAIR in tumor	*P*[a]	Blood donors, *n* (%)	HOTAIR in blood	*P*[a]
Age[b]	>67	39 (53)	1.31	0.96	41 (49)	4.66	0.31
	≤67	34 (47)	1.15		43 (51)	6.16	
Sex	Men	46 (63)	1.15	0.87	58 (69)	5.71	0.31
	Women	27 (37)	1.25		26 (31)	4.71	
TNM staging[c]	I–III	51 (69)	1.16	0.43	51 (60)	4.78	0.20
	IV	22 (31)	1.29		33 (40)	5.28	
Grade[d]	1–2	61 (84)	1.03	0.09	57 (69)	6.16	0.03
	3	12 (16)	3.36		26 (31)	3.74	
Tumor localization	Colon	36 (49)	1.15	0.76	43 (51)	4.78	0.58
	Rectum	37 (51)	1.25		41 (49)	5.73	
	Right colon	17 (23)	1.15	0.82	13 (15)	2.75	0.03
	Left colon	56 (77)	1.20		71 (85)	5.73	
Adjuvant therapy	Yes	57 (78)	1.31	0.13	45 (54)	5.17	0.32
	No	16 (22)	0.68		39 (46)	5.27	
MSI	Stable	37 (92)	1.23	NA[e]	55 (87)	4.14	NA[e]
	Unstable	3 (8)	0.62		8 (13)	3.54	

MSI, microsatellite instability; NA, not available.
[a]Mann–Whitney two-sided *U*-test.
[b]Groups were split by mean age of the study group.
[c]TNM stage system according to American Joint Committee on Cancer classification.
[d]Grade 1 stands for well-differentiated tumor, grade 3 stands for poorly differentiated tumor.
[e]*P* values not presented due to low number of patients with MSI unstable CRC.

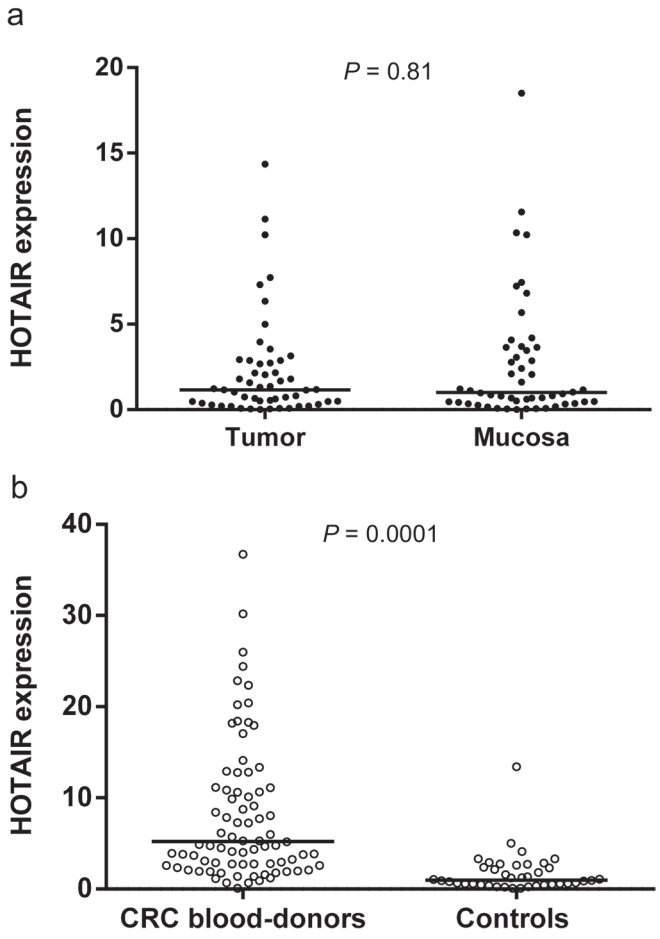

Fig. 1. Comparison of HOTAIR lncRNA levels between tumors ($n = 73$) and healthy mucosa ($n = 52$) (**a**) and difference between HOTAIR expression in blood of CRC blood donors ($n = 84$) and healthy controls ($n = 40$) (**b**).

HOTAIR relative expression in blood of CRC patients versus healthy controls

Significantly higher HOTAIR median relative expression was observed in PBMC of patients as compared with controls (5.22 versus 1.00, $P = 0.0001$; Figure 1B). In accordance with this significant difference, ROC analysis exhibited area under curve 0.87%, $P < 0.0001$ at 67% sensitivity and 92.5% specificity of tumor detection, respectively (Supplementary Material S3, available at *Carcinogenesis* Online). Patients with tumors localized in right colon had lower blood levels of HOTAIR as compared with patients with left colon tumors ($P = 0.03$). Patients with the poorly differentiated tumors (grade 3) showed lower median levels of HOTAIR than those with differentiated tumors (grade 1 and 2, $P = 0.03$; Table I). TNM staging did not show any association with HOTAIR expression (Table I). Since tissue and blood samples from patients were collected from different hospitals, we have also tested possible effect of the sampling center on the observed HOTAIR levels. No significant differences in HOTAIR expression between recruiting hospitals were observed (Supplementary Material S4, available at *Carcinogenesis* Online). In the plasma samples of CRC patients HOTAIR lncRNA levels never reached the limit of detection of $C_T = 35$. Only in three out of eight healthy controls, HOTAIR was present within the limit of detection. Therefore, HOTAIR expression in plasma was not further analyzed.

Correlation of HOTAIR relative expression between tumor tissue, healthy tissue and blood

In a subgroup of 26 patients sampled for all three biological specimens, significant correlation was observed between HOTAIR relative expression in tumors and paired healthy mucosa ($R = 0.68$, $P < 0.001$), mucosa and PBMC ($R = 0.70$, $P < 0.001$) and also between tumor and PBMC ($R = 0.41$, $P = 0.036$).

Relationship of HOTAIR relative expression with patients' prognosis

Univariate and multivariate Cox proportional hazards analysis was performed using main clinicopathological variables including TNM stage, histologic grade and HOTAIR expression. Cox analysis was calculated using all effects building, Breslow likelihood. We have arbitrarily subdivided patients according to their HOTAIR tumor/blood expression into two groups using best fit cutoff values based on

ROC analysis. Expression levels of HOTAIR above 0.7 for tumors and 4.4 for blood, respectively, were considered as 'high' HOTAIR relative expression groups. Univariate Cox proportional hazards analysis showed that patients with high HOTAIR expressions in tumors and patients with metastases had significantly higher risk of death. High HOTAIR tumor level together with the presence of metastases was negative prognostic factor according to multivariate Cox proportional hazards analysis (Table II). High HOTAIR levels detected in mucosa did not have any significant impact on death hazard. High HOTAIR level in blood was also associated with higher hazard ratio of death in univariate analysis, together with TNM IV stage. Multivariate analysis using main clinicopathological features such as TNM and histologic grade showed major effects of HOTAIR overexpression and TNM stage IV on the patients' prognosis (Table III). Cumulative proportion survival (Kaplan–Meier) showed significantly shorter survival in patients with high HOTAIR relative expression detected in tumor tissue (log-rank test $P = 0.030$; Figure 2A) as well as in blood (log-rank test $P = 0.008$; Figure 2B).

Discussion

HOTAIR lncRNA contributes to complex regulation of homeobox genes, important in the determination of spatiotemporal evolution of developing embryo. HOTAIR is therefore expressed at defined stages and sites of embryonal development (14). On the other hand, HOTAIR relative expression in adults have been documented in skin fibroblasts and its expression levels copy anteroposterior developmental axis, the highest expression being present in skin fibroblasts of the lower part of human body (14). HOTAIR is aberrantly overexpressed in several tumors, including breast, CRC and gastrointestinal stromal tumors (6,7,10). Although all these tumors exhibiting HOTAIR overexpression generally contain various amounts of fibroblast-like and inflammatory stromal components, a little is known, whether the HOTAIR overexpression is confined strictly to carcinoma cells or how extensively it could be associated with fibroblast-like or inflammatory stromal component of CRC tumors. Very recent contribution reports a semiquantitative determination of HOTAIR in formaldehyde-fixed paraffin-embedded tissues in breast cancer (15). Our present results show a lack of significant difference in HOTAIR overexpression between tumors containing mostly carcinoma cells and tumors containing predominantly stromal components. This finding suggests that high HOTAIR relative expression level is not specifically associated with either epithelial or fibroblast-like component of CRC carcinomas. This fact may have an important implication for the role of HOTAIR in tumor pathogenesis: HOTAIR relative expression levels do not seem to be directly associated with mutated carcinoma cell

Table II. Cox proportional hazard analysis: influence of HOTAIR tumor levels and different clinicopathological parameters on overall survival in tissue donor CRC patients

Univariate analysis	Category		Hazard ratio	Confidence interval		*P*
HOTAIR in tumor[a]	Low ($n = 37$)	High ($n = 36$)	4.43	1.02	19.19	0.046
Age	≤67 ($n = 36$)	>67 ($n = 37$)	1.96	0.77	4.97	0.157
Sex	F ($n = 27$)	M ($n = 46$)	1.25	0.40	3.85	0.703
TNM staging[b]	I–III ($n = 51$)	IV ($n = 22$)	3.61	1.447	9.017	0.006
Grade	1–2 ($n = 61$)	3 ($n = 12$)	3.42	0.46	25.67	0.232
Tumor localization	Colon ($n = 36$)	Rectum ($n = 37$)	1.43	0.54	3.81	0.470
	R. c.[c] ($n = 17$)	L. c.[c] ($n = 56$)	1.50	0.59	3.82	0.396
Stromal content	≤50% ($n = 33$)	>50% ($n = 40$)	0.70	0.20	2.50	0.582
Multivariate analysis	**Category**		**Hazard ratio**	**Confidence interval**		***P***
HOTAIR in tumor[a]	Low ($n = 37$)	High ($n = 36$)	4.46	1.02	19.79	0.048
TNM staging[b]	I–III ($n = 22$)	IV ($n = 22$)	3.87	1.49	10.07	0.006
Grade	1–2 ($n = 61$)	3 ($n = 12$)	0.60	0.20	1.77	0.352

First value in the 'Category' column serves as a reference value for hazard ratio calculation.
[a]Low and high HOTAIR groups were split by the cutoff value 0.7.
[b]TNM stage system according to American Joint Committee on Cancer classification.
[c]R. c. = right colon, L. c. = left colon.

Table III. Cox proportional hazard analysis: influence of HOTAIR blood levels and different clinicopathological parameters on survival in blood donor CRC patients

Univariate analysis	Category		Hazard ratio	Confidence interval		*P*
HOTAIR in blood[a]	Low ($n = 36$)	High ($n = 48$)	5.90	1.34	26.1	0.019
Age	≤67 ($n = 43$)	>67 ($n = 41$)	1.19	0.45	3.18	0.722
Sex	F ($n = 26$)	M ($n = 58$)	1.74	0.56	5.43	0.334
TNM staging[b]	I–III ($n = 51$)	IV ($n = 33$)	7.18	2.02	25.48	0.002
Grade	1–2 ($n = 57$)	3 ($n = 26$)	0.81	0.28	2.35	0.697
Tumor localization	Colon ($n = 43$)	Rectum ($n = 41$)	1.42	0.49	4.09	0.514
	R. c. [c] ($n = 13$)	L. c. [c] ($n = 71$)	NA[d]	NA[d]	NA[d]	NA[d]
Multivariate analysis	**Category**		**Hazard ratio**	**Confidence interval**		***P***
HOTAIR in blood[a]	Low ($n = 36$)	High ($n = 48$)	4.96	1.10	22.37	0.037
TNM staging[b]	I–III ($n = 51$)	IV ($n = 33$)	8.38	2.22	31.56	0.002
Grade	1–2 ($n = 57$)	3 ($n = 26$)	2.23	0.722	6.90	0.163

NA, not available. First value in the 'Category' column serves as a reference value for hazard ratio calculation.
[a]Low and high HOTAIR groups split by the cutoff value 4.4.
[b]TNM stage system according to American Joint Committee on Cancer classification.
[c]R. c. = right colon, L. c. = left colon.
[d]Data are not available due to low number of patients with right colon tumor.

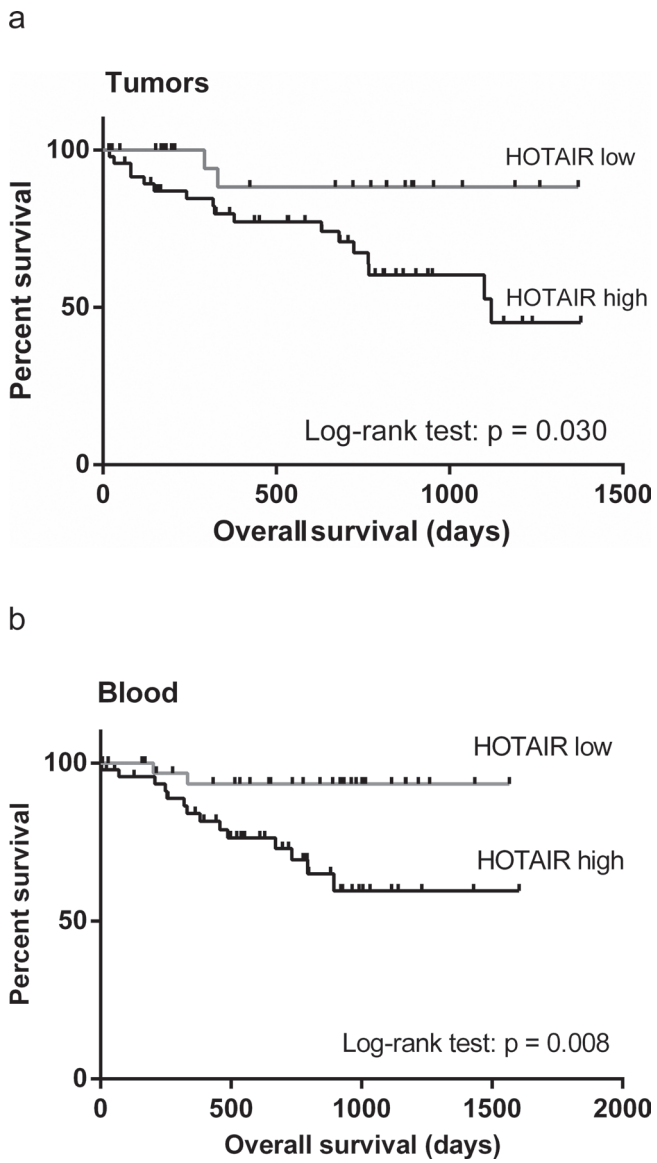

Fig. 2. Kaplan–Meier overall survival of 73 CRC tissue donors (**a**) and 84 CRC blood donors (**b**) stratified for low and high HOTAIR relative expressions.

clone from which carcinoma may originate, or with its clonal expansion. Most likely aberrant HOTAIR relative expression is rather a consequence of yet not fully identified (epigenetic) changes that force both epithelial and stromal (fibroblast-like eventually inflammatory) cells to upregulate HOTAIR within the tumor microenvironment. Mutual exosome transfer between breast carcinoma cells and stromal cells was documented (16). This may be one of many events that participate on the mutual communication between carcinoma cells and stroma and that can influence expression of prometastatic genes.

Interestingly, we did not observe any difference in median level of HOTAIR expression between tumors and adjacent healthy mucosas. Taking this observation with caution, we have controlled for potential role of relative expression evaluation method on the precision of reverse transcriptase qPCR data. Three different evaluation methods (i.e. use of reference sample with the lowest expression, percentage of total expression and median expression of control group) were applied to compare ROC curves. The data suggest that the evaluation method had no effect on the final observation.

We have observed that high HOTAIR levels in tumor tissue were associated with overall patients' prognosis, being related to higher risk of death and shorter survival. By multivariate Cox proportional hazard analysis we have further recorded that HOTAIR relative expression in tumor may serve as prognostic marker independent on major clinicopathological characteristics, except for metastasis status. Kogo *et al.* (10) reported that HOTAIR relative expression in tumors was an independent prognostic indicator of overall survival, with relative risk of death being 5.6 times higher in CRC patients with higher HOTAIR levels. This value is comparable with our observation of hazard ratio 4.4 in CRC patients with higher HOTAIR relative expression in tumors. Moreover, high HOTAIR levels were found to be related to poor prognosis in several other cancers such as pancreatic (8), nasopharyngeal (17), gastrointestinal stromal tumors (6), hepatocellular (9) and breast carcinoma (7).

Comparing HOTAIR levels measured in blood of CRC patients and healthy controls, patients had 4-fold higher levels of HOTAIR lncRNA. Moreover, these levels positively correlated with those in tumors and healthy mucosa. We assume therefore that HOTAIR expression in blood may be used as a surrogate to cancer target tissue and, additionally, levels of HOTAIR in blood might serve as a surrogate marker associated with CRC progression. High HOTAIR levels in PBMC were again associated with patients' overall survival, by increasing risks of death while decreasing survival period. However, HOTAIR relative expression in blood appeared to be prognostic factor in univariate Cox proportional hazard analysis only, whereas in multivariate analysis high HOTAIR relative expression was dependent on the presence of metastases, a phenomenon frequently observed in other cancers (7,8,17). Since HOTAIR blood levels were not yet reported for CRC patients and their survival, our novel contribution is related to the use of HOTAIR relative expression from non-invasively obtained surrogate tissue for an early estimation of the prognosis of CRC.

On the contrary to HOTAIR relative expression in primary tumors, its expression in PBMC was related to tumor localization. PBMCs from patients with ascendant (right) and transversal colon carcinomas exhibited lower HOTAIR relative expression compared with those with the tumors in descending (left) and sigmoideal parts of the bowel and rectum. Assuming that low HOTAIR is associated with better prognosis and *vice versa*, our data are in consent with the fact that colorectal tumors with chromosomal instability and aneuploidy, often localized in the left part of the bowel, exhibit an overall worse prognosis than those in the right colon (18). Thus, our study underlines the prognostic potential of HOTAIR expression level in tumor tissues of CRC patients, and for the first time also the prognostic value of HOTAIR expression in PBMCs. The study was conducted on ethnically homogeneous Central European population, which was well characterized for clinicopathological parameters and inspected for possible confounders. Moreover, all patients were incident, sampled for blood or tissue prior to any radio- or chemotherapy, to prevent possible interference with examined parameters. However, due to a relatively limited extent of the study population, the study should be considered as an exploratory. These facts underline the need to further validate above promising data on the replication set of CRC patients

In summary, our results show that HOTAIR overexpression both in blood and primary tumors is associated with unfavorable prognosis and it may identify patients that would require more intensive care concerning personally tailored preventive medical examinations and treatment.

Supplementary material

Supplementary Materials S1–S4 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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