Importance of miR-20a Expression in Prostate Cancer Tissue

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Abstract. *Background: MicroRNAs (miRNAs), which are endogenously expressed regulatory noncoding RNAs, have an altered expression in tumor tissues. MiRNAs regulate cancer-related processes such as cell growth and tissue differentiation, and therefore, might function as oncogenes or tumor-suppressor genes. The aim of our study was to assess the expression of mir-20a, let-7a, miR-15a and miR-16 in prostate cancer (PCa) and benign prostatic hyperplasia (BPH) tissue and to investigate the relation between the expression of miRNAs and the clinico-pathological features of PCa. Patients and Methods: The study group comprised 138 patients: 85 patients with BPH and 53 patients with PCa. The total RNA was isolated from the tissue specimen core and miRNA expressions were quantified using a real-time RT-PCR method (TaqMan MicroRNA Assays). U6snRNA was used for the normalization of the miRNA expression. Results: miR-20a expression was significantly higher in the group of patients with a Gleason score of 7-10 in comparison with the group of patients with a Gleason score of 0-6 (p=0.0082). We found no statistical differences in the miRNA expressions (mir-20a, let-7a, miR-15a and miR-16) in the PCa tissue samples in comparison with the BPH tissue samples. Conclusion: Our result shows that the more dedifferentiated PCa cells have a higher expression of miR-20a and this supports the oncogenic role of miR-20a in PCa carcinogenesis. The evaluation of miRNA expression could yield new information about PCa pathogenesis.*

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Prostate cancer (PCa) is the most commonly diagnosed cancer, and the second leading cause of cancer-related deaths of men in Western countries (1). Nevertheless, with regard to incidence, the molecular basis of PCa is insufficiently characterized. There is an increasing interest in the role of the new regulatory molecules, microRNAs (miRNAs), in cell processes, which could contribute to a better understanding of cancer pathology.

miRNAs are endogenously expressed, small non-coding RNAs, which regulate gene expression by the inhibition of the translation and/or decreasing of the stability of target mRNAs. Briefly, the miRNA expression in eukaryotic cells is realized in several steps. The miRNA gene is transcribed in the nucleus from the genomic DNA by RNA polymerase Pol II to produce a long transcript called a primary microRNA (pri-miRNA). This pri-miRNA is cleaved by a microprocessor complex containing the RNAse III enzyme Drosha and its cofactor Pasha (double-stranded RNA binding protein) into 70-100 nucleotide long precursor microRNA (pre-miRNA) with a typical hairpin structure. The pre-miRNA is exported by the transport protein exportin 5 into the cytoplasm, where the ~22 nucleotide miRNA duplex (miRNA/miRNA*) is excised from the pre-miRNA by RNAse III enzyme Dicer. Subsequently, one strand of the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which negatively regulates its target genes by preventing the production of their protein products.

Some miRNA genes are observed to be deregulated in cancer cells (2-6). The miRNAs can function both as oncogenes by down-regulating tumor-suppressor genes and as tumor-suppressor genes by down-regulating oncogenes (7). The reader is referred to reviews (2-5, 7, 8) and the public miRNA database available online at www.mirbase.org for more information on biogenesis, function and the role of miRNAs in carcinogenesis.

let-7 belongs to the first known miRNAs and is one of the most characterized. It was discovered in *Caenorhabditis*

Table I. *Comparison of expression levels of mirR-20a, let-7a, miR-15a and miR-16 between studied groups (BPH vs. PCa; organ-confined cancer (TNM 1c+2a+2b+2c) vs. advanced and metastatic (TNM 3a+3b+3c+1cM+2cM+3aM); Gleason score 0-6 vs. Gleason score 7-10).*

Group	N	miR-20a				let-7a			
		25%	Median	75%	p-Value	25%	Median	75%	p-Value
BPH	85	1.5476	3.8637	8.8766	0.3405	9.5137	17.2677	39.3966	0.5153
PCa	53	1.2746	3.5554	7.2100		8.0000	17.4550	30.9100	
Organ-confined cancer	41	1.2746	3.5554	.702100	0.9644	7.7275	12.8171	30.9100	0.0865
Advanced and metastatic	11	1.0353	3.6808	7.4643		15.4550	20.8215	83.2859	
Gleason score 0-6	27	1.1892	1.8661	3.8637	0.0082	6.9644	12.5533	27.8576	0.0707
Gleason score 7-10	20	3.5222	6.2467	15.0999		13.0431	18.0551	73.2024	
Group	N	miR-15a				miR-16			
		25%	Median	75%	p-Value	25%	Median	75%	p-Value
BPH	85	0.9013	1.6021	3.4343	0.8544	9.3827	19.6983	48.5029	0.3653
PCa	53	0.7738	1.6818	3.3636		6.1903	14.7230	42.2243	
Organ-confined cancer	41	0.7220	1.6586	3.3173	0.1033	4.6913	10.7779	42.2243	0.1708
Advanced and metastatic	11	1.6818	2.2974	3.4343		18.3792	20.8215	16.8507	
Gleason score 0-6	27	0.7738	1.6586	3.2043	0.0899	4.5315	12.3805	34.2968	0.1388
Gleason score 7-10	20	1.4315	2.9332	3.8025		8.7442	22.1075	90.5084	

elegans and its role in timing of stem cell division and differentiation was identified (9). let-7 is a member of the let-7 family and so far this family includes 10 mature sequences in humans (10). The let-7 family is often present in multiple copies in the genome. A letter is used to indicate isoforms with slightly different sequences. A subsequent number indicates the same sequences arising from different genomic locations (*e.g.* let-7a-1). In general, the function of let-7 is to promote the differentiation of cells.

The next most often studied group of miRNAs is the mir-17-92 cluster which contains miR-20a. Today there is evidence that the mir-17-92 cluster is involved in many types of human cancer. The amplification of the region containing this cluster was described in lymphoma (11, 12) and lung cancer (13). Loss of heterozygosity of these genes was recorded in breast cancer (14), hepatocellular carcinoma (15) and nasopharyngeal carcinoma (16-18). These results show that members of the mir-17-92 cluster could play a role both as oncogenes and tumor-suppressor genes.

A group of miRNAs with an antioncogenic role in cancerogenesis is the cluster miR-15 and miR-16. The role of these miRNAs was first identified in chronic lymphocytic leukemia (CLL). This cluster is located in the CLL frequently deleted or translocated region 13q14.3. Both miRNAs negatively regulate BCL2 at the post-transcriptional level (19).

Identification of deregulated miRNAs in cancer tissue can focus interest on these miRNAs and can help to determine their place in the cascade of carcinogenesis and finally they may become a promising targets for therapy.

The aim of our study was to asses the expression of let-7a, mir-20a, miR-15a and miR-16 in PCa tissue and benign prostatic hyperplasia (BPH) and investigate the relationship between expression of these miRNAs and clinicopathological features of PCa. We chose these miRNAs on the basis of previously published studies, based mainly on high throughput assays (20-23). This is the first direct study of these miRNAs in biopsied patient samples.

Patients and Methods

Patients. Our study group consisted of 138 patients who underwent a prostate biopsy between May 2006 and September 2008 in one of two Departments of Urology (the University Teaching Hospital in Pilsen and the University Teaching Hospital of the First Medical Faculty of Charles University in Prague). All the patients exhibited an elevated serum total prostate-specific antigen (tPSA) level and/or abnormal digital rectal examination. A previous biopsy was not an exclusion but had to have been performed at least three months prior to the study. The median age was 66.5 years (range 48-85 years). According to the histological verification, our group of patients was divided into 85 patients with BPH and 53 patients with PCa. The numbers of patients in compared groups are shown in Table I. The value of the Gleason score and TNM classification was not available for a small number of patients. Approval was obtained from the Institutional Ethics Committee and written informed consent from each patient. Patients who had had any transurethral manipulation, or radiotherapy, or who were on hormonal therapy, or had an indwelling catheter or acute urinary infection before the biopsy were excluded from the study.

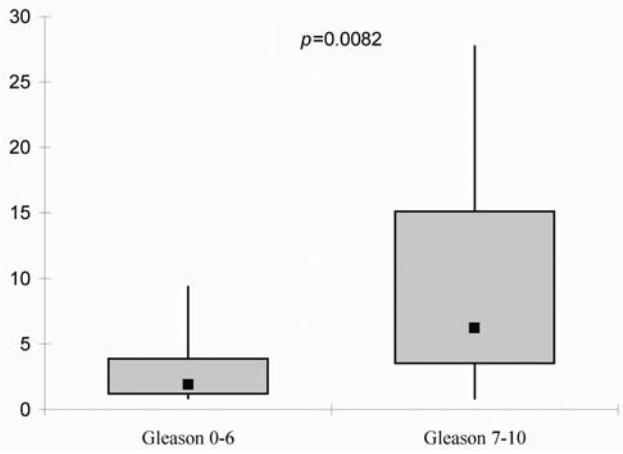

Figure 1. *Expression of miR-20a in PCa samples of Gleason score 0-6 and Gleason score 7-10. There was a significantly higher expression of miR-20a in Gleason score 7-10 group in comparison with Gleason score 0-6 group. The values shown in the figure are 10th and 90th percentiles (line), lower and upper quartiles (rectangle) and the median (small square) of miR-20a expression (relative copy number).*

Tissue samples. The tissue samples were obtained as a portion from a needle core bioptic sample, and were frozen at –70˚C until use.

Quantitative estimation of miRNA using RT real-time PCR. Total RNA was isolated from approximately 10 mg of tissue using a fastRNA Pro Green Kit (Q-BIOgene, Irvine, CA, USA). A quantitative estimation was performed by a RT real-time PCR method using TaqMan® MicroRNA Assays (Applied Biosystems, manufactured by Roche, Branchburg, NJ, USA). The two-step protocol requires reverse transcription with a miRNA-specific primer, followed by a real-time PCR with TaqMan® probes. The assays target only mature miRNAs, not their precursors. As a normalizer, RNU6B (U6snRNA), which is generally used in published studies (24-26) was used. Each sample and normalizer was assessed twice in parallel. The results of the expression were obtained as a relative copy number using the Ct value of the measured miRNA and Ct value of the normalizer (U6snRNA) by the following approach: The Ct values were corrected using calibrators for the elimination of differences between runs of the iCycler apparatus (Bio-Rad, Prague, Czech Republic) using the value of the expression: idCt=($Ct_{highest}$+1)–measured Ct, where $Ct_{highest}$ is the highest Ct of all the measured samples of the real marker *e.g*. miR-20a (27). This approach allows for an easy solution to the problem of samples with an unmeasurable expression (a value of 0 is assigned to the idCt of these samples) and a further advantage is the logic that a higher idCt means a higher expression (copy number). The final value of the relative copy number, R, was calculated using the following formula R=2(idCt miRNA–idCt normalizer)–dCt highest. Possible different $Ct_{highest}$ values of the measured miRNA and normalizer were corrected by the use of the $dCt_{highest}$ value which was calculated as the difference between $Ct_{highest}$ of the measured miRNA and the normalizer ($dCt_{highest}$=miRNA $Ct_{highest}$ –normalizer $Ct_{highest}$).

Statistical analysis was performed using the software SAS 8.02 (SAS Institute Inc., Cary, NC, USA). The statistical results were calculated by a Wilcoxon non-parametric two-sample test.

Results

We investigated differences in the expression of mirR-20a, let-7a, miR-15a and miR-16 in the BPH and PCa. All the measured values of expression and the P-values are summarized in Table I. We found no statistically significant differences in the expression of these miRNAs in BPH tissue in comparison with PCa tissue. Furthermore, we compared the expression in tumor tissue between different TNM stages of the disease. We compared the group of patients with organ-confined cancer (TNM 1c+2a+2b+2c) with those with a higher extent of the disease (advanced and metastatic; TNM 3a+3b+3c+1cM+2cM+3aM). We recorded no statistically significant differences in the expression of mirR-20a, let-7a, miR-15a and miR-16. Finally, we analyzed the differences in the expression of miR-20a, let-7a, miR-15a and miR-16 in two groups divided according to the degree of differentiation of PCa tissue, *i.e*. those with a Gleason score of 0-6 and those with a Gleason score of 7-10. We observed that the expression of miR20a was statistically significantly higher in the group with a Gleason score of 7-10 than in the group with a Gleason score of 0-6 (p=0.0082) (Figure 1). There were no differences in the expression of let-7a, miR-15a and miR-16 between these groups.

Discussion

In the past few years, research has revealed the role of miRNAs in the regulation of cell processes such as proliferation and apoptosis, as well as in the pathology of these processes. Despite the fact that the sequence of particular steps of the carcinogenesis of PCa is not known, it is evident that there is a deregulation of apoptosis and proliferation. We assessed the miRNAs which participate in these processes. We observed a higher expression of miR-20a in the group with a Gleason score of 7-10 than in the group with a Gleason score of 0-6. The Gleason score is an important variable describing the behavior of PCa and has been correlated with pathologic stage, metastasis and outcome (28). miR-20a is one of the members of the mir-17-92 cluster.

Sylvestre *et al*. described an overexpression of miR-20a in the human prostate cancer cell line PC3 using PCR (21). Volinia *et al*. recorded an up-regulation of miR-20a in PCa tissue using a microarray assay (22). The identified function of miR-20a is the modulation of the translation of the *E2F2* and *E2F3* mRNAs *via* binding sites in their 3'-untranslated region (21), this supports the oncogenic behavior of miR-20a. The same authors also observed antiapoptotic activity. miR-20a overexpression reduced apoptosis in the PC3 cell line (21). Our result supplements the previous findings and shows that the more dedifferentiated cancer cells (Gleason score 7-10) have a higher expression of oncogenic miR-20a.

It was reported that the other investigated miRNAs (let-7a, miR-15a and miR-16) have an antionco- or oncogenic function (3). The results of recent studies show that the *RAS* oncogene could be regulated at least by some members of the let-7 family (29). There is evidence that LIN28 plays an important role in the biogenesis of mature let-7 miRNA as a negative post-transcriptional regulator. High LIN28 levels prevent the processing of pri-let-7 by the microprocessor complex and also prevent pre-let-7 from turning into mature let-7 by dicer (10). This example also demonstrates that the assessment of pri-miRNA or pre-miRNA does not always corresponds with the level of mature miRNA. The miR-15a and miR-16 cluster is located in cancer frequently deleted or translocated region 13q14.3. Among the targets of miR-15a and miR-16, the antiapoptotic protein BCL2 was identified, which is overexpressed in many malignancies. Bonci *et al*. reported that in cancer cells of advanced prostate tumors, the miR-15a and miR-16 levels was significantly decreased (23). We did not observe differences in expression of miR-20a, let-7a, miR-15a and miR-16 between BPH and PCa. It should be noted that the behavior of BPH does not necessarily correspond with that of normal prostatic tissue; it was not possible for us to obtain normal prostate tissue.

In conclusion, our result extends the findings of previous studies and shows that the more dedifferentiated prostate cancer cells have a higher expression of miR-20a, supporting the oncogenic role of miR-20a in carcinogenesis of PCa.

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References

1 Jemal A, Siegel R, Ward E, Hao Y, Xu J and Thun MJ: Cancer statistics 2009. CA Cancer J Clin *59(4)*: 225-249, 2009.

2 Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer *6(4)*: 259-269, 2006.

3 Sassen S, Miska EA and Caldas C: MicroRNA: implications for cancer. Virchows Arch *452(1)*: 1-10, 2008.

4 Visone R and Croce CM: MiRNAs and cancer. Am J Pathol *174(4)*: 1131-1138, 2009.

5 Mirnezami AH, Pickard K, Zhang L, Primrose JN and Packham G: MicroRNAs: key players in carcinogenesis and novel therapeutic targets. Eur J Surg Oncol *35(4)*: 339-347, 2009.

6 Chan SH, Wu CW, Li AF, Chi CW and Lin WC: miR-21 microRNA expression in human gastric carcinomas and its clinical association. Anticancer Res *28(2A)*: 907-911, 2008.

7 Croce CM: Oncogenes and cancer. N Engl J Med *358(5)*: 502-511, 2008.

8 Carthew RW and Sontheimer EJ: Origins and mechanisms of miRNAs and siRNAs. Cell *136(4)*: 642-655, 2009.

9 Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR and Ruvkun G: The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature *403(6772)*: 901-906, 2000.

10 Roush S and Slack FJ: The let-7 family of microRNAs. Trends Cell Biol *18(10)*: 505-516, 2008.

11 He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ and Hammond SM: A microRNA polycistron as a potential human oncogene. Nature *435(7043)*: 828-833, 2005.

12 Venturini L, Battmer K, Castoldi M, Schultheis B, Hochhaus A, Muckenthaler MU, Ganser A, Eder M and Scherr M: Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. Blood *109(10)*: 4399-4405, 2007.

13 Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y and Takahashi T: A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res *65(21)*: 9628-9632, 2005.

14 Eiriksdottir G, Johannesdottir G, Ingvarsson S, Björnsdottir IB, Jonasson JG, Agnarsson BA, Hallgrimsson J, Gudmundsson J, Egilsson V, Sigurdsson H and Barkardottir RB: Mapping loss of heterozygosity at chromosome 13q: loss at 13q12-q13 is associated with breast tumour progression and poor prognosis. Eur J Cancer *34(13)*: 2076-2081, 1998.

15 Lin YW, Sheu JC, Liu LY, Chen CH, Lee HS, Huang GT, Wang JT, Lee PH and Lu FJ: Loss of heterozygosity at chromosome 13q in hepatocellular carcinoma: identification of three independent regions. Eur J Cancer *35(12)*: 1730-1734, 1999.

16 Shao J, Li Y, Wu Q, Liang X, Yu X, Huang L, Hou J, Huang X, Ernberg I, Hu LF and Zeng Y: High frequency loss of heterozygosity on the long arms of chromosomes 13 and 14 in nasopharyngeal carcinoma in Southern China. Chin Med J (Engl) *115(4)*: 571-575, 2002.

17 Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F and Croce CM: Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA *99(24)*: 15524-15529, 2002.

18 Poliseno L, Pitto L, Simili M, Mariani L, Riccardi L, Ciucci A, Rizzo M, Evangelista M, Mercatanti A, Pandolfi PP and Rainaldi G: The proto-oncogene *LRF* is under post-transcriptional control of MiR-20a: implications for senescence. PLoS One *3(7)*: e2542, 2008.

19 Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, Taccioli C, Zanesi N, Garzon R, Aqeilan RI, Alder H, Volinia S, Rassenti L, Liu X, Liu CG, Kipps TJ, Negrini M and Croce CM: MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci USA *105(13)*: 5166-5171, 2008.

20 Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL and Visakorpi T: MicroRNA expression profiling in prostate cancer. Cancer Res *67(13)*: 6130-6135, 2007.

21 Sylvestre Y, De Guire V, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, Ferbeyre G and Chartrand P: An E2F/miR-20a autoregulatory feedback loop. J Biol Chem *282(4)*: 2135-2143, 2007.

22 Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC and Croce CM: A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA *103(7)*: 2257-2261, 2006.

23 Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, Bartucci M, Muto G, Peschle C and De Maria R: The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med *14(11)*: 1271-1277, 2008.

24 Choong ML, Yang HH and McNiece I: MicroRNA expression profiling during human cord blood-derived CD34 cell erythropoiesis. Exp Hematol *35(4)*: 551-564, 2007.

25 Corney DC, Flesken-Nikitin A, Godwin AK, Wang W and Nikitin AY: MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res *67(18)*: 8433-8438, 2007.

26 Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS and Sung JJ: Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut *58(10)*: 1375-1381, 2009.

27 Peters IR, Helps CR, Calvert EL, Hall EJ and Day MJ: Cytokine mRNA quantification in duodenal mucosa from dogs with chronic enteropathies by real-time reverse transcriptase polymerase chain reaction. J Vet Intern Med *19(5)*: 644-653, 2005.

28 Stamey TA, McNeal JE, Yemoto CM, Sigal BM and Johnstone IM: Biological determinants of cancer progression in men with prostate cancer. JAMA *281(15)*: 1395-1400, 1999.

29 Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D and Slack FJ: RAS is regulated by the let-7 microRNA family. Cell *120(5)*: 635-647, 2005.

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