ORIGINAL ARTICLE

Immuno-histochemical Evaluation of α -methylacyl-CoA Racemase Expression in Colorectal Carcinoma

Shahram Bagheri, Roya Ghafghazi*

Department of Pathology, Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran

Study Area: Ahvaz, Iran

Coordinates: 31°19'13"N; 48°40'09"E

Key words: Alpha-methylacyl-CoA racemase, Colorectal cancer, Colon, Immunohistochemistry

Ethic code: Ahvaz Jundishapur University of Medical Sciences ethics committee (IR.AJUMS.REC.1397.359).

Abstract

A case-control study aimed to evaluate the diagnostic value of Alpha-methylacyl-CoA racemase immunoreactivity in patients with colorectal neoplasia where paraffin blocks of colorectal malignant and normal tissue of patients were used. Histological findings were compared with AMACR immunohistochemistry results. The in silico analysis was carried out to find the prognostic significance of AMACR expression levels in colon cancers. A total of 30 patients with colorectal cancer (46 biopsy specimens, 16 normal tissues and 30 cancerous tissues) were evaluated. The mean level of staining extent in patients was 87% (range 30 to 100%). More than 90% of patients had a strong positive reaction and only 2 patients (6.7%) had a weakly response. The immunoreactivity in half of the cases was 2. There is no significant relationship between the type of cancer and gender. Similarly, the intensity and extent of staining in different types of colorectal cancer were not significantly different. In addition, the expression of the AMACR marker did not have a significant relationship with the age of the patients as well as the gender of the patients. Of the 16 normal colon samples, only 2 cases were weakly positive. Sensitivity and specificity were calculated as 100% and 87 %, respectively. The upregulation of AMACR was significantly associated with a decreased survival rate. The findings acknowledge the potential capacity of the AMACR marker as a diagnostic tool in identified colon neoplasia.

Introduction:

Colorectal cancer is the second leading cause of cancer-related death in the world. The 5-year survival rate is very low and more than 50% of newly diagnosed cases are in advanced stages of the disease (McCormick et al., 2003). Advanced colorectal cancers are often associated with metastasis to the peritoneum, lymph nodes and other organs (Gayet et al., 2001). African-American races are at a higher risk of developing this type of cancer, with mortality rates higher than those of other races (Gayet et al., 2001). Diets rich in fruits, vegetables, calcium, and vitamin D have associated with decreasing the risk of colorectal cancer. In contrast, people with less physical activity, heavy smokers and alcoholic consumers are at higher risk (Gayet et al., 2001; Kinzler et al., 1991). It has been shown that the consumption of red meat products also has a significant correlation with the incidence of colorectal cancer. Meat and dairy products are rich in fatty acids, which require the

Alpha-methylacyl-CoA racemase (AMACR) / P504S enzyme to oxidize them. AMACR is located in the mitochondria and peroxisome and is essential for fatty acids Beta-oxidation (Rubin et al., 2002).

Upregulation of AMACR has been observed in many pre-neoplastic conditions as well as many cancers such as prostate adenocarcinoma, colorectal carcinoma, hepatocellular carcinoma and renal cell carcinoma (Xu et al., 2014; Molinié et al., 2006; Shi et al., 2007; Lakis et al., 2010). Immunohistochemical assessment of AMACR was initially used to diagnose prostate adenocarcinoma. However, further studies have shown that more than half of the colon adenocarcinomas are also immunoreactive against AMACR antibody (Rosai, 2011). Moreover, The increased expression of AMACR in pro malignant and malignant colorectal lesions indicates its potential role in its carcinogenesis process (Lin et al., 2007). The AMACR up-regulation induced by nutritional factors may play a major role in

*Corresponding Author: ghafghazi.Roya@gmail.com

ORIGINAL ARTICLE

regulating cell proliferation by increasing the expression of acyl-CoA oxidase. Investigators had shown that the AMACR expression induction thorough the complete gene transfection caused Prostate cancer cell expansion. (Lin et al., 2007). It has also been suggested that the AMACR up-regulation may cause its carcinogenic effect by providing energy for neoplastic cells (Shi et al., 2007).

Till date, the diagnostic value of AMACR immunopositivity in the diagnosis of colorectal neoplasia has not been well studied. The present study was aimed to evaluate the immunohistochemical expression of AMACR in colorectal neoplasia, as well as its relationship with histological grade and colorectal staging.

Results:

Study Design: this observational study was carried out on patients admitted to the Pathology department of Imam Khomeini hospital, Ahvaz, Iran having history of neoplasia, history of Chemotherapy or Radiotherapy. Patients suffering from secondary colorectal cancer were excluded from the study. Patients information were extracted from the files. Immunohistochemical assessment: the specimens were stained by H&E and after pathologic confirmation, the following steps were performed to prepare immunohisto-chemical slides.

Tissue Preparation: first, the samples were dehydrated by ascending ethanol (70, 95 & 99%). Then, the samples clarified by xylene. The sample molding was carried out by paraffin.

Cutting: The tissue sections were prepared in a thickness of 4 to 5 microns.

Immunohistochemical staining: de-paraffinization was initially performed by floating samples in Xylene. It was then put in sequential descending ethanol concentrations (99, 95 & 70%). In order to stop the endogenous Peroxide activity, three sections were incubated for 10 minutes in hydrogen peroxide 5% and then washed with water. Subsequently, Antigen retrieval was done by incubating the samples in 0.01 M sodium citrate for 1 min at 98°C. The endogenous peroxide activity was stopped by a 10% normal (Goat) serum for five minutes. The primary antibody at a dilution of 1:200 was added and incubated for 1 hour at 4°C and then washed with TBS. After that, the samples were incubated with a secondary antibody at 1: 500 dilution for 45 min and washed with TBS. Chromogenic reactivity performed by diaminobenzidine and contrasting staining was done by Hematoxylin. At least 1000 cells were counted in each slide and the percentage of stained cells was determined. Color intensity scored as negative (0), poor (+1), moderate (2+) and strong (+ 3). The staining percentage was scored as below: 0 to 5% (score 0), 20-6% (score 1), 40- 21% (score 3), 80-61% (grade 4), and 81 to 100% (score 5).

Insilica analysis:

Oncomine analysis: the expression level of AMACR gene in the different types of colorectal cancers was determined using the Oncomine database (Rhodes *et al.*, 2004). To do that, we have used cancer Vs. normal datasets. P valued less than 0.01 selected as a threshold to reduce the false discovery rate. The fold change of gene expression level in different colorectal subtype was compared.

Kaplan-Meier analysis: the prognostic value of the AMACR gene in colorectal cancer was analyzed using the PROGgeneV2 - Pan Cancer Prognostics Database, an expression based survival analysis database for many types of cancers (Goswami & Nakshatri, 2014). The hazard ratio (HR) with 95% confidence intervals and log-rank p value was reported.

Statistical analysis: all data were analyzed by descriptive statistics including mean, standard deviation, frequency, and percentage. Proportions were compared using the Chi-Square test. All statistical analysis was performed using SPSS version 19. A P-value less than 0.05 considered significant.

Results:

In this study, 30 patients with colorectal cancer (46 biopsy specimens, 16 normal tissues, and 30 neoplastic tissues) were evaluated. The demographic information and other relevant characteristics are in Table-1. More than 56% of the tumors were completely differentiated. Nearly half of the patients were on stage PT3 and No (Table-1).

The AMACR immunohistochemical expression information is shown in Table 2. Accordingly, the mean staining percentage was 87% (range 30 to 100%). More than 90% of patients had a strong positive response (Fig.-1), and only 2 patients (6.7%) had a poor positive response. The staining intensity was +2 in about half of the cases (Table - 2,3).

The relationship between different types of colorectal cancer with studied variables is shown in Table-3. The diagnostic value of AMACR to differentiate the malignant from benign colon tissue was calculated and it was shown that this marker had a sensitivity of 100% and a specificity of 87% (Table-4).

Figure-1: The strong expression of AMACR marker in colon malignant tissue.

Table-1: Patient characteristics

Characteristics	Mean(range)	SD	Frequency(%)
Age	64.56(33-89)	±15.4	
Gender	Male - 17(56.7%)	Female- 13(43.3%)	
Diagnosis			
Adenocarcinoma Colon-		8 (26.7%)	
Adenocarcinoma Rectum		6 (20%)	
Adenocarcinoma Sigmoid		14 (46.7%)	
Adenocarcinoma cecum		2 (6.7%)	
Grade			
Well-Differentiated	17 (56.7%)	Moderately-	
Differentiated	13 (43.3%)		
Stage			
PT2 No	4 (13.3%)	PT3 No	13 (43.3%)
PT4 No	3 (10%)	PT3 N1a	3 (10%)
PT3 N1b	2 (6.7%)	PT3 N1c	1 (3.3%)
PT3 N2a	2 (6.7%)	PT3 N3b	2 (6.7%)

Table-2: Immunochemical staining signature of AMACR marker in patients with colorectal cancer

Characteristics	Mean(range)	SD	Frequency(%)
Extent	87(30-100)	±19.1	
Intensity			
1 Plus	8(26.7%)	2 Plus	14(46.7%)
3 Plus	8(26.7%)		
Positivity			
Strongly-Positive	28(93.3%)	Weakly-Positive	2(6.7%)

Table 3: Relationship between studied variables with type of colorectal cancer

Parameter	*Colon	*Rectome	*Sigmoid	*Secom	Total
Gender	$X^2=3.8, df=3, p>0.282$				
Male	n 6	3	6	2	17
	% 35.3%	17.6%	35.3%	11.8%	100.0%
Female	n 2	3	8	0	13
	% 15.4%	23.1%	61.5%	0.0%	100.0%
Grade	$X^2=2.58, df=3, p>0.46$				
Well-Differentiated	n 3	3	10	1	17
	% 17.6%	17.6%	58.8%	5.9%	100.0%
Moderately-Differentiated	n 5	3	4	1	13
	% 38.5%	23.1%	30.8%	7.7%	100.0%
Intensity	$X^2=2.83, df=3, p>0.83$				
1+	n 2	1	4	1	8
	% 25.0%	12.5%	50.0%	12.5%	100.0%
2+	n 4	4	6	0	14
	% 28.6%	28.6%	42.9%	0.0%	100.0%
3+	n 2	1	4	1	8
	% 25.0%	12.5%	50.0%	12.5%	100.0%
Positivity	$X^2=1.68, df=3, p>0.64$				
Strongly Positive	n 8	5	13	2	28
	% 28.6%	17.9%	46.4%	7.1%	100.0%
Weakly-Positive	n 0	1	1	0	2
	% 0.0%	50.0%	50.0%	0.0%	100.0%

The mRNA expression of AMACR in different types of colorectal was extracted from TCGA colorectal dataset. Based on that, AMACR gene expression upregulated in all studied types of CRC (Fig-2). The prognostic significance of AMACR

expression in terms of overall survival (OS), Metastasis progression and relapse rate were evaluated based on GSE28814 datasets. Although the AMACR up-regulation significantly decreased the OS, it has no significant effect on metastasis progression and as well as relapse rate (Fig- 3-5).

Table-4: Accuracy of the AMACR marker in the diagnosis of colon malignancy

Sensitivity:	1.000;	95% confidence interval- 0.88 to 1.00
Specificity:	0.87;	95% confidence interval- 0.61 to 0.98
+ve Predictive Value:	0.93;	95% confidence interval- 0.79 to 0.99
-ve Predictive Value:	1.000;	95% confidence interval-0.76 to 1.00

Figure-2: Gene expression levels of AMACR in different types of colorectal cancer

Figure-3: AMACR expression significantly affect the survival rate

Figure-4: AMACR expression effects on Metastasis progression

ORIGINAL ARTICLE

Figure-5: AMACR expression effects on Relapse rate

Discussion:

AMACR protein is an enzyme that plays an important role in beta-oxidation of branched fatty acids and fatty acid derivatives. This catalytic enzyme is the conversion of 2R fatty acids into S-stereoisomers (Ferdinandusse *et al.*, 2000, 2001; Jiang *et al.*, 2003; Schmitz *et al.*, 1995). Increasing the expression of this protein in prostate cancer suggested its role in cancer biology, and it has become a routine tool in diagnosing prostate cancer (Xu *et al.*, 2000; Martignoni *et al.*, 2007; Molinie *et al.*, 2006; Went *et al.*, 2006). Further studies showed that the increased expression AMACR enzyme in multiple cancers (Went *et al.*, 2001). The potential benefits of AMACR evaluation in the diagnosis of dysplasia, especially in the gastrointestinal tract, were extensively investigated (Dorer & Odze, 2006).

The immunopositivity rate of AMACR in 30 patients with colorectal cancer was 100%, which means that all patients with colorectal cancer highly expressed AMACR markers. The staining intensity was more than 2 plus in more than half of the cases, and more than 90% of the patients showed a strong positive reaction to the AMACR marker. These findings were in line with Shukla *et al.* (2017) who reported from 12 studied adenoma cases; 25% were negative, 50% had shown weak reactivity, and 25% moderate reactivity. Jiang *et al.* (2003) in a study with 242 colorectal cancer patients (including 176 CRC, 38 adenomas, and 28 hyperplastic polyps) showed that AMACR expression was very low or negative in the normal colon tissue, while 76% of Well-differentiated colon cancer and 75% moderately differentiated carcinomas had strong and moderate expression. Only 4% of hyperplastic polyps had AMACR expression. Further, Lee *et al.* (2006), studied gastric normal tissue and showed that non-neoplastic tissues were only AMACR positive in 4.5% of cases. However, the expression of this marker in the gastric carcinoma was 83.3% and in the stomach dysplasia 51.5%. Silico analysis at the molecular level had also confirmed our findings, based on that all colorectal neoplasia had shown AMACR up-regulation. Therefore, since the expression of AMACR in the normal tissue was negative, its positive expression had a

diagnostic value. We evaluated the diagnostic value of AMACR in the differentiation of the malignant from benign colon tissue. The AMACR had a sensitivity of 100% and a specificity of 87% in identifying colorectal malignancy. Our findings have shown that AMACR marker expression was not related to the disease stage. Similarly, the findings of Shukla *et al.* (2017) were shown that the expression of AMACR did not correlate with the disease stage according to the AJCC Guide.

Also, Marx *et al.* (2008) in a study of over 1300 patients with colorectal showed that AMACR expression was observed in 1074 patients (81.7%). They also evaluated the AMACR immunopositivity value in predicting prognosis and indicated that its expression was not related to the clinical outcomes of patients. In the contrary, we have shown that the AMACR up-regulation may significantly decrease the OS.

Conclusively, overall the findings of the present study, in completing previous findings, showed that the AMACR enzyme has widely expressed in colorectal cancer cells. These findings acknowledge the potential capacity of the AMACR marker as a diagnostic tool in identified colon neoplasia. The main limitation of the study was the low sample size.

Acknowledgment: We thank all our colleagues in Imam Khomeini Hospital, Ahvaz, Iran

References:

- Dorer, R. & Odze, R.D. (2006): AMACR immunostaining is useful in detecting dysplastic epithelium in Barrett's esophagus, ulcerative colitis, and Crohn's disease. *Am. J. Surg. Pathol.*, 30(7):871-877.
- Ferdinandusse, S., Denis, S., Ijlst, L., Dacremont, G., Waterham, H.R. & Wanders, R.J. (2000): Subcellular localization and physiological role of alpha-methylacyl-CoA racemase. *J. Lipid Res.*, 41(11):1890-1896
- Ferdinandusse, S., Overmars, H., Denis, S., Waterham, H.R., Wanders, R.J. & Vreken, P. (2001): Plasma analysis of di- and trihydroxycholestanic acid diastereoisomers in peroxisomal alpha-methylacyl-CoA racemase deficiency. *J. Lipid Res.*, 42(1):137-141.
- Gayet, J., Zhou, X.P., Duval, A., Rolland, S., Hoang, J.M., Cottu, P. & Hamelin, R. (2001): Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. *Oncogene*, 20(36):5025-5032.
- Goswami, C.P. & Nakshatri, H. (2014): PROGgeneV2: enhancements on the existing database. *BMC Cancer*, 14:970
- Jiang, Z., Fanger, G.R., Woda, B.A., Banner, B.F., Algate, P., Dresser, K., Xu, J. & Chu, P.G. (2003): Expression of alpha-methylacyl-CoA racemase (P504s) in various malignant neoplasms and normal tissues: a study of 761 cases. *Hum. Pathol.*, 34(8):792-796
- Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P. & McKechnie, D. (1991): Identification of FAP locus genes from chromosome 5q21. *Science*, 253(5020):661-665.
- Lakis, S., Papamitsou, T., Panagiotopoulou, C., Kotakidou, R. &

- Kotoula, V. (2010): AMACR is associated with advanced pathologic risk factors in sporadic colorectal adenomas. *World J. Gastroenterol.*, 16(20):2476-2483.
- Lee, W.A. (2006): α -Methylacyl-CoA-Racemase Expression in Adenocarcinoma, Dysplasia and Non-Neoplastic Epithelium of the Stomach. *Oncology*, 71(3-4):246-250.
- Lin, A., Weiser, M.R., Klimstra, D.S., Paty, P.B., Tang, L.H., Al-Ahmadie, H., Hoo Park, S., Guillem, J.G., Temple, L., Wong, W.D., Gerald, W.L. & Shia, J. (2007): Differential expression of α -methylacyl-coenzyme A racemase in colorectal carcinoma bears clinical and pathologic significance. *Hum. Pathol.*, 38(6):850-856.
- Martignoni, G., Brunelli, M., Gobbo, S., Remo, A., Ficarra, V., CossuRocca, P., Pea, M., Chilosì, M., Menestrina, F. & Cheng, L. (2007): Role of molecular markers in diagnosis and prognosis of renal cell carcinoma. *Anal. Quant. Cytol. Histol.*, 29(1):41-49.
- Marx, A., Simon, P., Simon, R., Mirlacher, M., Izbicke, J.R., Yekebas, E., Kaifi, J.T., Terracciano, L. & Sauter, G. (2008): AMACR expression in colorectal cancer is associated with left-sided tumor localization. *Virchows Arch.*, 453(3):243-248.
- McCormick, D., Kibbe, P.J. & Morgan, S.W. (2002): Colon cancer: prevention, diagnosis, treatment. *Gastroenterol. Nurs.*, 25(5): 204-211.
- Molinie, V., Balaton, A., Rotman, S., Mansouri, D., De Pinieux, I., Homsì, T. & Guillou, L. (2006) : Alpha-methyl CoA racemase expression in renal cell carcinomas. *Hum. Pathol.*, 37(6):698-703.
- Rhodes, D.R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A. & Chinnaiyan, A.M. (2004): ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia*, 6(1):1-6.
- Rosai, J. (2011): **Rosai and Ackerman's Surgical Pathology** - 2 Volume Set 10th Edition. Pub. by: Elsevier, London UK: P.-2892.
- Rubin, M.A., Zhou, M., Dhanasekaran, S.M., Varambally, S., Barrette, T.R., Sanda, M.G., Pienta, K.J., Ghosh, D. & Chinnaiyan, A.M. (2002): α -Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *J. Am. Med. Assoc.*, 287(13):1662-1670.
- Schmitz, W., Albers, C., Fingerhut, R. & Conzelmann, E. (1995): Purification and characterization of an alpha-methylacyl-CoA racemase from human liver. *Eur. J. Biochem.*, 231(3):815-822.
- Setchell, K.D., Heubi, J.E., Bove, K.E., O'Connell, N.C., Brewsaugh, T., Steinberg, S.J., Moser, A. & Squires, R.H. Jr. (2003): Liver disease caused by failure to racemize trihydroxycholestanic acid: gene mutation and effect of bile acid therapy. *Gastroenterol.*, 124(1):217-232.
- Shi, X., Gong, E. & Wu, X. (2007): Alpha-methylacyl-CoA racemase/P504S overexpression in colorectal carcinoma is correlated with tumour differentiation. *Appl. Immunohistochem. Mol. Morphol.*, 15(2):175-180.
- Shukla, N., Adhyam, A.K. & Rath, J. (2017): Expression of Alpha-Methylacyl-Coenzyme A Racemase (AMACR) in Colorectal Neoplasia. *J. Clin. Diagn. Res.*, 11(4):EC35-EC38.
- Went, P.T., Sauter, G., Oberholzer, M. & Bubendorf, L. (2006): Abundant expression of AMACR in many distinct tumour types. *Pathology*, 38(5):426-432.
- Xu, B., Cai, Z., Zeng, Y., Chen, L., Du, X., Huang, A., Liu, X. & Liu, J. (2014): α -Methylacyl-CoA racemase (AMACR) serves as a prognostic biomarker for the early recurrence/metastasis of HCC. *J. Clin. Pathol.*, 67(11):974-979.
- Xu, J., Stolk, J.A., Zhang, X., Silva, S.J., Houghton, R.L., Matsumura, M., Vedvick, T.S., Leslie, K.B., Badaro, R. & Reed, S.G. (2000): Identification of differentially expressed genes in human prostate cancer using subtraction and microarray. *Cancer Res.*, 60(6):1677-1682.