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2014

Abdel-Rahman , W M , Nieminen , T T , Shoman , S , Eissa , S & Peltomaki , P 2014 , ' Loss of p15(INK4b) Expression in colorectal cancer is Linked to Ethnic Origin ' , Asian Pacific journal of cancer prevention , vol. 15 , no. 5 , pp. 2083-2087 . https://doi.org/10.7314/APJCP.2014.15.5.2083

http://hdl.handle.net/10138/161873
https://doi.org/10.7314/APJCP.2014.15.5.2083

RESEARCH ARTICLE

Loss of $p15^{INK4b}$ Expression in Colorectal Cancer is Linked to Ethnic Origin

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Abstract

Colorectal cancers remain to be a common cause of cancer-related death. Early-onset cases as well as those of various ethnic origins have aggressive clinical features, the basis of which requires further exploration. The aim of this work was to examine the expression patterns of $p15^{INK4b}$ and SMAD4 in colorectal carcinoma of different ethnic origins. Fifty-five sporadic colorectal carcinoma of Egyptian origin, 25 of which were early onset, and 54 cancers of Finnish origin were immunohistochemically stained with antibodies against $p15^{INK4b}$ and SMAD4 proteins. Data were compared to the methylation status of the $p15^{INK4b}$ gene promotor. $p15^{INK4b}$ was totally lost or deficient (lost in ≥50% of tumor cell) in 47/55 (85%) tumors of Egyptian origin as compared to 6/50 (12%) tumors of Finnish origin (p=7e-15). In the Egyptian cases with $p15^{INK4b}$ loss and available $p15^{INK4b}$ promotor methylation status, 89% of cases which lost $p15^{INK4b}$ expression were associated with $p15^{INK4b}$ gene promotor hypermethylation. SMAD4 was lost or deficient in 25/54 (46%) tumors of Egyptian origin and 28/48 (58%) tumors of Finnish origin. 22/54 (41%) Egyptian tumors showed combined loss/deficiency of both $p15^{INK4b}$ and SMAD4, while $p15^{INK4b}$ was selectively lost/deficient with positive SMAD4 expression in 24/54 (44%) tumors. Loss of $p15^{INK4b}$ was associated with older age at presentation (>50 years) in the Egyptian tumors (p=0.04). These data show for the first time that $p15^{INK4b}$ loss of expression marks a subset of colorectal cancers and ethnic origin may play a role in this selection. In a substantial number of cases, the loss was independent of SMAD4 but rather associated with $p15^{INK4b}$ gene promotor hypermethylation and old age which could be related to different environmental exposures.

Keywords: Colorectal cancer - immunohistochemistry - methylation - $p15^{INK4b}$ - SMAD4

Asian Pac J Cancer Prev, **15 (5)**, 2083-2087

Introduction

$p15^{INK4b}$ (*CDKN2B*) is a tumor suppressor gene located, together with two other related genes *ARF* and $p16^{INK4a}$ (*CDKN2A*) within a 35 kb stretch on chromosome 9p21. The INK4a/ARF/INK4b locus is deleted in a variety of tumors including melanoma, pancreatic adenocarcinoma, glioblastoma, certain leukemias, non-small cell lung cancer, and bladder carcinoma (Kim Sharpless, 2006; Nakamura et al., 2011). The binding of the INK4 proteins to the cyclin dependent kinases CDK4 and CDK6 abrogates the binding of these kinases to D-type cyclins, thus inhibiting CDK4/6-mediated phosphorylation of retinoblastoma (pRb) protein and its family members. Hypophosphorylated Rb-family proteins potently bind E2F transcription factors to exert a G1 cell-cycle arrest (Kim and Sharpless, 2006). Deregulation of pRb pathway is common in human cancers, but direct alterations of the pRb protein or its closely associated molecules are rarely observed in colorectal cancer apart from the infrequent loss of $p16^{INK4a}$ expression associated with promoter methylation (Cheng et al., 2006; Joensuu et al., 2008). Colorectal cancers, however, undertake a more drastic upstream manoeuvre to deregulate the pRb-mediated cell cycle control through eliminating the growth inhibitory response to TGF-β (Lu et al., 1995; Lampropoulos et al., 2012). In normal cells, binding of the TGF-β to its receptor TGF-βRII, causes phosphorylation of several SMAD proteins, such as SMAD3 and SMAD2 which form a heterodimeric complex with SMAD4. The SMAD3/SMAD4 (or SMAD2/SMAD4) dimer then migrates to the nucleus, where it teams up with MIZ-l to induce expression of the $p15^{INK4b}$. TGF-β signalling also relieves $p15^{INK4b}$ from the *MYC*-induced repression by down regulating the *MYC* gene expression (Warner et al., 1999; Seoane et al., 2001). More recently, SMAD/STAT3 signaling pathway was shown to play a role in epithelial-to-mesenchymal transition during colorectal carcinogenesis (Zhu et al., 2013) and single nucleotide polymorphism (SNIP) variations within one of the

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SMADs (*SMAD7*) have influenced the susceptibility to colorectal cancers (Nassiri et al., 2013).

We recently found remarkable frequent hypermethylation of the *p15^{INK4b}* gene promoter in colorectal carcinoma of Egyptian origin (Nieminen et al., 2012). Conversely, hypermethylation of *p15^{INK4b}* was reported mainly in glial tumors, leukemias, myelodysplasia (Esteller et al., 2001), hepatocellular carcinoma (Zekri Ael et al., 2013) and, more recently, in peripheral blood of leukemia patients (Bodoor et al., 2014), but was not a common finding in colorectal carcinoma of Western origin (Cheng et al., 2006; Nieminen et al., 2012). Interestingly, *p15^{INK4b}* methylation was detected in 68% of colorectal cancer specimens of Chinese origin (Xu et al., 2004) and in 26% colorectal cancers from Japan (Ishiguro et al., 2006). Egyptian colorectal carcinoma is surprisingly young age disease with high proportion of rectal and advanced stage cancers. The *p15^{INK4b}* methylation data could explain these clinical differences and link them to exposure to environmental toxins, since gene methylation may be related to different environmental exposures.

Here, we characterized sporadic colorectal cancers of Egyptian and Finnish origins for expression of *p15^{INK4b}* and its closely related upstream protein SMAD4 by immunohistochemistry staining and correlated the results with the clinico-pathological and gene methylation data available on this series.

Materials and Methods

Patients and samples

This study was performed on a consecutive series of 55 Egyptian carcinoma and 54 cancers of Finnish origin (Table 1). These cases were selected from a bigger series (Nieminen et al., 2012) according to the availability of immunohistochemistry tissue sections. Samples were collected, from formalin fixed paraffin embedded tissue blocks of surgical resection specimens as explained previously (Nieminen et al., 2012). DNA was extracted from paraffin-embedded specimens by standard techniques. Mutation screening, microsatellite instability (MSI), methylation analyses and p53 immunohistochemistry were performed in previous studies (Joensuu et al., 2008; Nieminen et al., 2012). The work was conducted at Helsinki under the approval of the institutional review boards of the Helsinki University Central Hospital.

Table 1. Pathological and Molecular Characteristics of the Egyptian *vs* Western tumors

	Egypt	Finn sporadic
No. of tumors in the original series	69	61
No. included in the current study	55	50
No. included in methylation analysis	43	34
Age range (average)	18-78 (54.8)	52-95 (73.6)
Gender (M:F)	1.23:1	0.5:1
Tumor site (Rt : Lt : Rectal)	15:21:33	35:17:4*
MSI frequency	19/61a (31%)	16/61 (26%)
p53 stabilization	43/68 (63%)	29/57 (51%)
Nuclear β-catenin	28/68 (41%)	45/58 (78%)
TSGMP phenotype (≥5 genes methylated)	22/43 (51%)	14/51 (27%)

*Variation in the numbers or denominator used for calculating percentages resulted from missing data. Molecular data were generated in previous study (Nieminen et al., 2012). MSI, microsatellite instability; TSGMP, tumor suppressor gene methylator phenotype

Immunohistochemistry

Four-micrometer sections from formalin-fixed paraffin-embedded tissues were de-waxed and re-hydrated to distilled water then sections were subject to heat-induced target retrieval in 1 mM ethylenediaminetetraacetic acid (EDTA) buffer pH 8.0 for 5 minutes at 750 W followed by 5 minutes at 450 W in a microwave oven. After cooling, the slides were washed in Tris-buffered saline/Tween 20 ph 7.2 and subsequent staining steps were performed manually with the Dako EnVision+ System, Peroxidase (DAB), according to manufacturer's instructions (Dako, Glostrup, Denmark). Additionally, after blocking endogenous peroxidase activity, and prior to incubation with the primary antibody, the sections were incubated with 10% normal (non-immune) goat serum (Dako, Glostrup, Denmark) for 30 minutes. The primary antibodies were: anti *p15^{INK4b}* mouse monoclonal antibody clone15P06 used at dilution 1:25 and anti SMAD4 rabbit monoclonal antibody clone EP618Y at dilution 1:200. Both antibodies were purchased from Abcam (Cambridge, UK). Primary antibody incubation was for 2 hours at room temprature. Paired tumor and normal mucosa were in the same section and the normal tissues were used as internal reference for evaluation of staining results.

Interpretation of staining results

Interpretation of staining results was performed by experienced histopathologist (W M A-R) SMAD4 staining was cytoplasmic in normal mucosa and neoplastic cells. Tumors showing positive staining in more than 50% of neoplastic cells were considered positive, tumors showing staining in less than 50% of neoplastic cells were considered 'deficient' while tumors showing staining of less than 2% of neoplastic were considered negative. The cut-off level of 50% was according to Sakellariou et al (Sakellariou et al., 2008). p15^{INK4b} expression was nuclear and a scoring scale similar to the one described above with a 50% cut-off level was employed according to the published literature (Oda et al., 2005; Endo et al., 2011).

Statistical analysis

Fisher's exact probability test was used to evaluate differences between groups. Analyses were performed using MS Excel and/or VassarStats Web-based statistical program http://faculty.vassar.edu/lowry/VassarStats.html. All reported p values were two-tailed and p values < 0.05 were considered significant.

Results

Immunohistochemistry p15^{INK4b} and SMAD4

The analyses showed that p15^{INK4b} was totally lost or deficient (lost in ≥50% of tumor cell) in 47/55 (85%) tumors of Egyptian origin as compared to 6/50 (12%) tumors of Finnish origin (p=7e-15). SMAD4 was lost or deficient in 25/54 (46%) tumors of Egyptian origin and 28/48 (58%) tumors of Finnish origin. 22/54 (41%) Egyptian tumors showed combined loss/deficiency of both

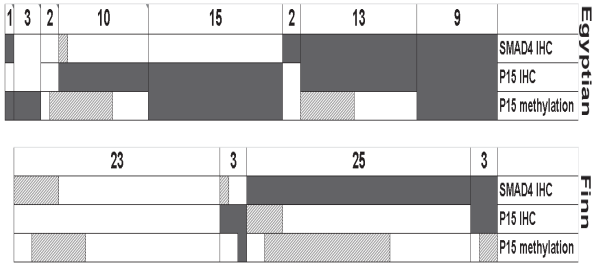

Figure 1. Diagrammatic Comparison between the SMAD4 and p15^{INK4b} Results from the Egyptian Tumors (top) and Finnish Tumors (bottom). Black boxes under the immunohistochemistry heading indicate deficient/lost expression, while the black boxes under methylation indicate methylated p15^{INK4b} promotor. The hatched/grey boxes indicate that data were not available for these cases. The number of cases in each category is indicated on the top

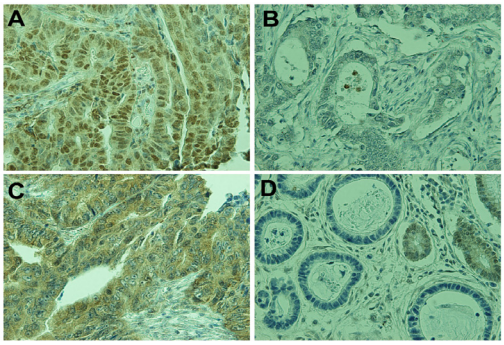

Figure 2. p15^{INK4b} and SMAD4 Immunohistochemistry. A, positive nuclear staining of p15^{INK4b} in carcinoma; B, loss of p15^{INK4b} staining in carcinoma; C, positive cytoplasmic staining of SMAD4 in carcinoma; D, loss of SMAD4 expression in carcinoma compared to normal mucosa (upper right corner). Original magnification ×100

Table 2. Expression p15^{INK4b} in Relation to Clinicopathological and Molecular Features of Egyptian Colorectal Cancer

		p15^{INK4b} positive n/(%)	p15^{INK4b} reduced or negative n/(%)	p value
Gender	Male	4 (13%)	27 (87%)	0.7
	Female	4 (17%)	20 (83%)	
Age	≤50	8 (23%)	27 (77%)	0.04
	>50	0 (0%)	20 (100%)	
Differentiation	Good/moderate	4 (12%)	28 (88%)	1
	Poor	3 (13%)	20 (87%)	
Stage	Early (I&II)	3	14	1
	Late (III& IV)	5	30	
Location	Right	3	9	0.7
	Left/rectal	7	36	
MSI status	MSI	3	12	0.7
	MSS	5	33	
p53	Negative	2	22	0.3
	Stabilized	6	24	
β-catenin	Membranous	7	22	0.1
	Nuclear	2	24	

p15^{INK4b} and SMAD4, while p15^{INK4b} was selectively lost/deficient with positive SMAD4 expression in 24/54 (44%) (Figure 1, 2).

Correlation of p15^{INK4b} expression and methylation status

The available *p15^{INK4b}* promotor methylation data showed more frequent methylation in the Egyptian series (28/42; 67%) compared to the Finnish series (1/33; 3%; p=7e-9). In the Egyptian cases with *p15^{INK4b}* loss and available *p15^{INK4b}* promotor methylation status, 89% of cases which lost *p15^{INK4b}* expression were associated with *p15^{INK4b}* gene promotor hypermethylation (Figure 1).

Relationship between p15^{INK4b} expression and pathological and molecular features

Table 2 shows p15^{INK4b} expression in relation to the clinico-pathological and molecular features of the Egyptian tumors. Significant correlation was found between older age at presentation (>50 years) and the loss of *p15^{INK4b}* (p=0.04). No significant relation was found between p15^{INK4b} expression and microsatellite instability status, p53 expression, or β-catenin localization.

Discussion

Prompted by our finding of remarkable p15^{INK4b} promoter methylation in colorectal cancers of Egyptian origin (Nieminen et al., 2012), we have analysed the immunohistochemical expression of p15^{INK4b} and SMAD4 in colorectal cancers of Egyptian and Western origins with the purpose to exploit these markers in diagnosis and personalized medicine. The results of the present study lead us to speculate that the loss of p15^{INK4b} protein expression marks the development of subsets of colorectal cancers of Eastern origins. This is supported by the methylation data on colorectal cancers of Chinese (Xu et al., 2004) and Japanese origin (Ishiguro et al., 2006). SMAD4 expression was lost or deficient in around half of the tumors examined of both Egyptian and Finnish origin consistent with the published literature (Royce et al., 2010; Ahn et al., 2011). SMAD4 is a potential upstream inducer of *p15^{INK4b}* (see introduction). Hence, some cases of p15^{INK4b} loss could be attributed to SMAD4 deficiency, but, SMAD4 deficiency cannot be considered sufficient to explain the remarkable loss of p15^{INK4b} in the Egyptian tumors since it was not associated with similar p15^{INK4b} loss in the Finn cancers.

The loss of *p15^{INK4b}* expression was reported in non-epithelial malignancies including leukemias, malignant peripheral nerve sheeth tumors, meningioma, and melanoma (Herman et al., 1997; Teofili et al., 2000; Simon et al., 2001; Endo et al., 2011). Furthermore, in support of our present findings, immunohistochemical expression studies of p15^{INK4b} in epithelial cancers showed substantial loss in a lineage specific fashion. Sakellariou and co-workers reported p15^{INK4b} loss in more than 70% of advanced gastric cancers, especially the intestinal subtype (Sakellariou et al., 2008). Consistent with our data, no correlation was observed between p15^{INK4b} and pathological or survival data apart from tendency to affect male gender and distal location within the stomach (Sakellariou et al., 2008). In cutaneous squamous cell carcinoma, p15^{INK4b} protein expression was absent in the majority of cases (69%) but, there was no significant relationship between clinicopathologic variables of the patients (age, sex and tumor grade) and p15^{INK4b} protein expression (Moad et al., 2009). More recently, Holm et al reported loss of p15^{INK4b} in 82% of vulvar squamous cell

carcinomas which correlated significantly with increased invasiveness. However, they could not establish $p15^{INK4b}$ as independent prognostic markers (Holm et al., 2013).

Interestingly, the loss $p15^{INK4b}$ in the Egyptian series was associated with its gene promoter methylation and with old age at onset suggesting a potential causal relationship. While tumor suppressor promoter methylation is known to increase with age (Fraga et al., 2007), aging alone seems insufficient to explain our data given the higher average age at onset of colorectal cancer (Table 1) but significantly less frequent methylation in the Finnish series (Figure 1). Paun et al 2010 demonstrated that environmental toxins such as smoking were associated with gene methylation in the normal rectal mucosa and with the presence of colorectal adenomas. These methylated genes were potentially involved in early stages of adenoma formation and the authors speculated that the observed epigenetic alterations in these markers may be caused in part by the effects of smoking and/or age (Paun et al., 2010). Exposures to environmental toxicants and toxins might cause epigenetic changes (O'Hagan, 2013; Coppede et al., 2014; Senut et al., 2014) and it is clear that many different adverse environmental factors are likely to exist in the East compared to the West as discussed previously (Nieminen et al., 2012). Our data, together with the available literature (Belinsky et al., 2004; Marsit et al., 2006) suggest a link between environmental exposures, epigenetic changes and cancers development, which remains to be confirmed in experimental models and large series of clinical samples.

Acknowledgements

This work was supported by the Terry Fox Foundation (W.M.A.-R.) and Sigrid Juselius Foundation (W.M.A.-R. & P.P.), the Finnish Cancer Organizations (P.P.), Academy of Finland grant no. 257795 (P. P.), Biocentrum Helsinki (P. P.), and European Research Council grant no. FP7-ERC-232635 (P. P.). The authors thank Saila Saarinen for expert technical assistance.

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