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IGFBP7 plays a potential tumor suppressor role against colorectal carcinogenesis with its expression associated with DNA hypomethylation of exon 1*

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Abstract: Insulin-like growth factor binding-protein-7 (IGFBP7) was obtained from our previous colonic adenocarcinoma (CRC) and normal mucosa suppression subtraction hybridization (SSH) cDNA libraries. By RT-PCR and immunohistochemistry, we found that IGFBP7 was overexpressed in CRC tissue compared to normal tissue. However, our in vitro experiments performed in 10 CRC cell lines showed that IGFBP7 expressed only in SW480 and Caco2 cell lines, which implied an underlying reversible regulatory mechanism. Using methylation-specific PCR (MSP) and bisulfite sodium PCR (BSP), we found that its expression was associated with DNA hypomethylation of exon1. This was further supported by the in vitro study which showed restored IGFBP7 expression after demethylation agent 5-aza-2'-deoxycytidine treatment. Correlation analysis between IGFBP7 expression and prognosis indicated that overexpression of IGFBP7 in CRC tissue correlated with favourable survival. Investigation of the functional role of IGFBP7 through transfection studies showed that IGFBP7 protein could inhibit growth rate, decrease colony formation activity, and induce apoptosis in RKO and SW620 cells, suggesting it a potential tumor suppressor protein in colorectal carcinogenesis. In conclusion, our study clearly demonstrated that IGFBP7 plays a potential tumor suppressor role against colorectal carcinogenesis and its expression is associated with DNA hypomethylation of exon 1.

Key words: IGFBP7 (Insulin-like growth factor binding-protein-7), Colorectal cancer, Tumor suppressor protein, Methylation
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IGFBP7 (insulin-like growth factor binding-protein-7), also named mac25, tumor-derived adhesion factor (TAF), prostacyclin-stimulating factor (PSF), or insulin-like growth factor binding-protein-related protein 1 (IGFBP-rP1), was originally cloned as a gene whose expression was decreased in meningioma cell lines (Murphy *et al*., 1993). IGFBP7 exhibited low affinity for the known IGFBP ligands, IGF-I, and IGF-II (Kim *et al*., 1997), indicating its individual characters differ from that of other members of the IGFBP family.

There were increasing evidences which showed that IGFs and IGFBPs could be included in the panel of specific markers used for histopathological diagnosis and serological surveillance procedures in various malignancies (Zumkeller, 2001). Recently, research focused on the relation between IGFBP7 and tumor was also conducted. IGFBP7's expression has been reported in many tumors. However, the expression pattern varies with tumor types. Both

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up-regulated expression (How *et al*., 1999) and down-regulated expression (Komatsu *et al*., 2000; Watson *et al*., 2002) of IGFBP7 is observed in different types of cancer. Interestingly, while for breast cancer and prostate cancer, both up- and down-regulation of IGFBP7 have been reported (Sprenger *et al*., 1999; Degeorges *et al*., 1999; Seth *et al*., 2003; Bieche *et al*., 2004). However, till now, in vitro studies on the role of IGFBP7 in cancer cells reached the same conclusion that IGFBP7 plays a negative role on the growth of cancer cells, including breast cancer cells (Wilson *et al*., 2002), human prostate cancer cells (Mutaguchi *et al*., 2003), human cervical carcinoma cells (HeLa) and murine embryonic carcinoma cells (P19), as well as osteosarcoma cells (Saos-2) (Kato, 2000). Together with its derivation as a senescence associated gene, IGFBP7 is more like a tumor suppressor gene. While as to colorectal carcinoma (CRC), although there have been some research focused on IGFBP7 in CRC (Adachi *et al*., 2001), which showed that the expression of IGFBP7 in invading tumor cells correlates with poor prognosis in human colorectal cancer, suggesting that IGFBP7 may play a positive role in tumor progression, comprehensive work on IGFBP7 in CRC is lacking.

Our team devoted to research on the role IGFBP7 plays in colorectal carcinoma since the cDNA fragments of IGFBP7 were selected from colonic adenocarcinoma and normal mucosa cDNA subtraction libraries by suppressive subtractive hybridization (SSH) (Luo and Lai, 2001). By using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, we confirmed the overexpression of IGFBP7 in CRC tissue, compared to the paired normal tissue (Shao *et al*., 2004). However, our in vitro experiments performed in colorectal cancer cell lines showed that the expression of IGFBP7 was only detectable in SW480 and Caco2 cell lines, but not in most colorectal cancer cell lines including HCT8, SW1116, RKO, HT29, COLO205, Hce8693, CW2 and SW620. The different expression pattern of IGFBP7 in colorectal carcinoma and colorectal cancer cell lines stimulated us to find the exact regulatory mechanism. Mutaguchi *et al*.(2003) showed that demethylation reagent 5-aza-dC could restore IGFBP7 mRNA expression in prostate cancer cell lines. Their finding implied that aberrant DNA methylation might play an important role in regulating IGFBP7 expression although no more detailed information on the methylation of IGFBP7 was obtained. In our study, the methylation status of the intriguing CpG island of IGFBP7 in 46 tissue samples was determined by methylation-specific PCR (MSP) method as previously described (Herman *et al*., 1996). As a result, the methylation of IGFBP7 exon 1 was much lower in cancers than in matched normal tissues (28/46, 60.9% in cancers versus 37/46, 80.6% in normal mucosa, $P<0.05$). The expression level of IGFBP7 was negatively correlated with the methylation status in colorectal cancers ($r=-0.210$, $P=0.044$ or $r=-0.299$, $P=0.004$ as indicated by RT-PCR and immunostaining, respectively). As for the CRC cell lines, the methylation difference between IGFBP7-positive and -negative cell lines was statistically significant ($P<0.001$). Hypermethylation of IGFBP7 exon 1 was negatively associated with IGFBP7 expression ($r=-0.935$, $P<0.001$). Depending on the methylation status of the exon 1 region, SW480 and Caco2, the 2 IGFBP7-positive cell lines, could be clustered into one group and other IGFBP7-negative cell lines into other groups. Again, the expression of IGFBP7 was restored after 5-aza-dC treatment in SW620, COLO205 and HT29 cell lines, suggesting that methylation of IGFBP7 was the key mechanism muted in expression.

The different expression pattern of IGFBP7 in CRC tissue and cell lines also spurred us to think about another question, whether it is enough to conclude the functional role of IGFBP7 in colorectal cancer merely by its expression pattern? To further explore its exact role, we then analyzed the correlation between expression level of IGFBP7 and prognosis. Intensity of immunochemical staining was designated as negative (0), positive (1), strong positive (2) and the percentage of positive cells was scored as less than 5% (0), 5%~25% (1), 26%~50% (2), 51%~75% (3) or over 75% (4) of cells stained. These values were multiplied together to provide composite score for IGFBP7 expression. According to the composite scores, 12 (5.5%), 145 (66.5%) and 61 (28.0%) carcinomas had scores of 0, 1, 2, respectively. We designated the 0 group as IGFBP7 negative, the 1 and 2 groups IGFBP7 positive. Patients with positive IGFBP7 expression had better survival compared with patients with negative IGFBP7 expression ($P<0.05$). The composite scores of IGFBP7

expression were identified as the independent factors for prognosis using multivariate Cox proportional hazard model. However, our finding contrasted with the results of Adachi *et al*.(2001), who found that high expression of IGFBP7 in invasive tumor cells was associated with poor prognosis. This discrepancy may be due to the difference in the immunohistochemical scoring. We used the composite score to evaluate the expression of IGFBP7, which seems to be one of the most promising and accurate scoring systems currently defined (Spano *et al*., 2005).

We also conducted research on the function of IGFBP7 protein in RKO and SW620 cell models. The proliferation of IGFBP7 transfectants was significantly slower than that of the control vector-transfectants and parental cells ($P<0.01$). Colony-forming activity in the IGFBP7 transfectants was significantly lower than the control empty vector transfectants (mean 12/well vs 59/well for RKO, mean 19/well vs 59/well for SW620, $P<0.05$ respectively). Size of the clone is smaller in IGFBP7-RKO and IGFBP7-SW620 transfectants in comparison to the control, though the difference is more significant in RKO cells, which confirmed that IGFBP7 could reduce the proliferation rate of these two types of CRC cells.

FCM analysis followed by both FITC-conjugated annexin V/PI staining and PI staining showed that apoptotic cells were prominent in cell cultures 48 h post-transfection with IGFBP7, but relatively rare in control vector-transfected cells. Chromatin condensation and margination were also observed by transmission electron microscopy after transfection of IGFBP7 into RKO cells and SW620 cells. This process may involve the upregulation of caspase-3 in RKO cells.

Our above finding indicated that IGFBP7 overexpression in colorectal cancer is associated with DNA hypomethylation of exon 1 and correlated with favourable prognosis. Our study suggested that IGFBP7 plays a potential tumor suppressor role in colorectal carcinogenesis. It is interesting that IGFBP7 was overexpressed in CRC tissue but exerted a potential tumor suppressor role. A possible explanation could be that IGFBP7 may be a part of the self-defense mechanism against tumor, whose secretion may slow down the growth of cancer cells as a protection to the human body. Interestingly, proteins such as maspin, which was identified to be strong, with almost uniform IHC staining, as well as high expression in an expanded set of pulmonary carcinomas, has to date been considered a suppressor protein (Welsh *et al*., 2003). From these studies, we can know that only depending on the expression pattern of a gene in cancer is far from yielding a conclusion on the exact role the gene plays. To make a conclusion on the gene's exact role, experiments from different respect should be performed.

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