RESEARCH ARTICLE

Functional Analysis of B7-H3 in Colonic Carcinoma Cells

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Abstract

   B7-H3 is a newly discovered member of the B7/CD28 superfamily which functions as an important T-cell immune molecule. It has been reported recently that B7-H3 is highly expressed in many cancer cells, the data indicating that it may be a regulation factor contributing to tumor-resistance. In our study, we used bioinformatics to identify differentially expressed genes between colonic cancer cells and normal colonic cells, aiming to analyze mechanisms and identify sub-pathways closely related to progression, with the final aim of finding small molecule drugs which might interfere this progression. We found that ajmaline is one related factor which may enhance self-immunity in colon carcinoma therapy and B7-H3 plays important roles with regard to immunoreactions of colonic cancer cells. All the results indicate that H7-B3 is a favorable prognostic biomarker for colon carcinomas, providing novel information regarding likely targets for intervention.

Keywords: B7-H3 - colon carcinoma - GSEA - small molecule mimic - focal adhesion

Introduction

   B7-H3 (B7 homologue 3), a newly found member of B7/CD28 superfamily (Shin et al., 2010), exists as two isoforms: B7-H3 VC, which contains one IgV- and IgC-like domain, and B7-H3 VCVC, which contains two such domains. The latter represents the predominant B7-H3 molecule detectable in various human tissues. Both B7-H3 isoforms are shown to decrease the proliferation and cytokine production induced by TCR activation of human T cells in vitro (2005). Performing as an important molecule in T cell immune response, B7-H3 stimulates proliferation of both CD4+ and CD8+ T cell, enhances the induction of cytotoxic T cells and selectively stimulates interferon gamma (IFN-gamma) production in the presence of T cell receptor signaling (Chapoval et al., 2001). The expression of B7-H3 and B7-H2 by Human nasal epithelial (HNE) cells is a couple of potential co-stimulatory signals, through which these cells may interact with activated mucosal T lymphocytes (Saatian et al., 2004), and that is similarly as the known human leukocyte antigen (HLA) and B7 homolog family co-stimulatory molecules, which expressed on the epithelial cells of the human respiratory tract and affects the cellular differentiation and cytokines.

   Recently, there are a lot of reports on the highly association of B7-H3 over-expression with kinds of cancers. For example, B7-H3 is over-expressed by all six non-small-cell lung cancer (NSCLC) cell lines on both mRNA and protein level. (Sun et al., 2006; Xu et al., 2010) And in prostate cancer, expression level of B7-H3 was correlated with pathologic indicators of aggressive cancer as well as clinical outcome. B7-H3 is uniformly and aberrantly expressed by adenocarcinomas of the prostate,

high-grade prostatic intraepithelial neoplastic, and four prostate cancer cell lines and expressed by benign prostatic epithelia (Roth et al., 2007; Zang et al., 2007; Yuan et al., 2011). Also in breast cancer, B7-H3 mRNA expression was detected 39% in primary breast tumors but not in normal breast tissues (Ahmed, 2010). B7-H3 expression was highly correlated to sentinel lymph node and overall number of lymph nodes with metastasis (Arigami et al., 2010). And finally in gastric cancer, blood specimens contained significantly more copies of B7-H3 mRNA than those from healthy volunteers (Biglarian et al., 2010). The 5-year survival rate was significantly lower in patients with high B7-H3 expression than with low expression (Arigami et al., 2011). Expect these poor prognostic which B7-H3 linked to, the opposite effect has been observed in other cancers, such as in human oral squamous cell cancer (Yang et al., 2008; Nygren et al., 2011). So the function of B7-H3 is needed to be further unearthed individually in miscellaneous cancers.

   In colorectal cancer, endothelial B7-H3 expression was also significantly associated with poor outcome and there was observation that B7-H3 expression in tumor-associated vasculature and fibroblasts. In rectal cancer patients, the only significant association was between fibroblast B7-H3 expression and shorter metastasis-free survival. These indicate that nuclear B7-H3 might be involved in colon cancer progression and metastasis, and suggest that nuclear B7-H3 could become a useful prognostic marker in colon cancer (Lupu et al., 2006; Ingebrigtsen et al., 2012). However, the multiple changes trigged by B7-H3 over-expression in colon cancer and the panorama view of its function pathway and mechanism remains to be clarified. Therefore, we tried our way to denote the functions of B7-H3 in this article.

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Materials and Methods

Expression Profile of Colonic Cancer Cells and Normal Colonic Cells

To probe the differences between colonic cancer cells and normal colonic cells and to clarify the roles of B7-H3 in colonic cancer cells, we collected colorectal carcinoma cells and the normal cells and analyzed their expression profiles with gene chip. Firstly, we searched proper sample expression data from GEO (Gene Expression Omnibus) database and chose GSE23878 as an object. This chip set contains 35 colonic carcinoma chips, 23 normal colonic chips. The platform information is GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. We downloaded the original CEL file as well as the denotation files of this platform.

Extraction of Differentially Expressed Genes

After obtaining the original chip data, we analyzed the chip data via R software (v.2.13.0) (Team, 2008). The whole chip data is classified into two classes: colon carcinoma cells and normal cells. RMA (Robust Multichip Averaging) method (Irizarry et al., 2003) was at first being applied to normalize the data on different chips, and the B7-H3 gene expression profile was obtained. Then limma (Smyth, 2004), a set of linear regression model software kit, were used to compare the differential expressions on different classes of chips so the differential expressed genes between colon carcinoma cells and normal cells were obtained.

GO Cluster with B7-H3 Influence

To chase the changes between differentially expressed genes on cell level and cluster their functions, we searched the database Gene Otology. And then we used DAVID to cluster the differential expressed genes according to the GO Intracellular component, biological process and molecular function (Huang da et al., 2009; Huang da et al., 2009) to get the influence information of differentially expressed genes on cells. Finally, we focused on the classes B7-H3 located and obtained the information about the affection of B7-H3 on cells.

Biological Pathways Influenced by B7-H3

In order to unearth the influence of B7-H3 on colon carcinoma cells, we focus on the biological pathway level. All the metabolism and non-metabolism pathways got from authority pathway database KEGG PATHWAY DATABASE were used as DAVID network materials for KEGG PATHWAY cluster analysis (Huang da et al., 2009; Huang da et al., 2009) and then the changed pathways in colon carcinoma betray themselves. Finally, those pathways linked to B7-H3 or in which B7-H3 located were focused to understand the molecular mechanisms B7-H3 performed its functions.

Unearthing Small Molecules Facilitating B7-H3 Functions

The connectivity map (CMAP) database, storing whole genomic expression profiles under small active molecular inferences, contains 6,100 classes of small molecular interference experiments and 7,056 expression profiles. (Lamb et al., 2006) We analyzed differential expressed genes between normal and colon carcinoma cells, contrast these genes with genes responded to small molecular interference in CMAP database, hoping to find some small molecular associated with those differentially expressed genes between normal cells and bladder carcinoma cells. The differentially expressed genes, located in the same cluster with B7-H3 or highly associated with B7-H3, between normal and colon carcinoma cells were classified into up regulated and down regulated, from which respectively 500 most eminent probes were chosen for GSEA analysis, and then compared to the differentially expressed genes after small molecular treatment, and finally enrichment values was gain. These enrichment values, varied between -1 and 1, determine the similarity. The more the value is closer to 1, the more similar between these genes, i.e. the small molecular can imitate the effects of B7-H3. And on the contrary, if the value is closer to -1, it illustrates that these small molecular can interrupt the effects of B7-H3.

DAB immunohistochemistry staining analysis of Colonic Cancer Cells and Normal Colonic Cells

A total of 104 cases of colon cancer specimens and clinical data were obtained from colon cancer patients with intact follow-up data who subjected to radical resection in the cancer center. The specimens were constructed from formalin-fixed, paraffin-embedded tissues. The expression of in tumor cells matched with adjacent noncancerous tissue was examined with immunohistochemistry technique. Additionally, immunofluorescence staining technique was used to investigate the molecular mechanism by analyzing expression of B7-H3.

Results

Expression Difference of B7-H3

As a critical factor linked to immunity, B7-H3 is overexpressed in several kinds of cancers. To probe if these differentially expression condition also occurred in colon carcinoma, we extract the expression profile about B7-H3 from chips. After normalization treatment, we got the profile of B7-H3 expression as Figure 1.

To probe the differentially expressed genes trigged by B7-H3 changes, we applied canonical t-test method to both colon carcinoma and normal cells' gene expression profiles and obtained the differentially expressed genes in colon carcinoma cells. After t-test to all the genes, associated
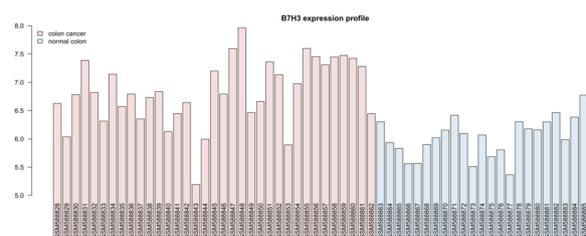
Figure 1. The Gene Expression Profile of B7-H3 Expressed in Colon Carcinoma and Normal Colon Cells. The characters under colon bars are the chip number of GEO

Table 1. Changed Biological Pathways in Colon Carcinoma

Term	Benjamini
hsa04510:Focal adhesion	0.002387
hsa05200:Pathways in cancer	0.002731
hsa04110:Cell cycle	0.007004
hsa04514:Cell adhesion molecules (CAMs)	0.010973
hsa04115:p53 signaling pathway	0.011895
hsa04662:B cell receptor signaling pathway	0.012952
hsa04914:Progesterone-mediated oocyte maturation	0.016172
hsa04666:Fc gamma R-mediated phagocytosis	0.018162
hsa04012:ErbB signaling pathway	0.018822
hsa05222:Small cell lung cancer	0.019102
hsa00230:Purine metabolism	0.019299
hsa05212:Pancreatic cancer	0.020673
hsa05211:Renal cell carcinoma	0.026616
hsa05213:Endometrial cancer	0.028989
hsa05214:Glioma	0.030032
hsa04360:Axon guidance	0.030626
hsa05218:Melanoma	0.032362
hsa00071:Fatty acid metabolism	0.032734
hsa04660:T cell receptor signaling pathway	0.03404
hsa04540:Gap junction	0.03484
hsa05215:Prostate cancer	0.03484

Table 2. Small Molecules Associated with B7-H3 Function

cmap name	enrichment	p
tanespimycin	-0.509	0
LY-294002	-0.4	0
trichostatin A	-0.344	0
diloxanide	0.914	0.00004
thioridazine	-0.504	0.00004
citalopram	0.854	0.00062
bretylium tosilate	0.853	0.0007
ciclopirox	0.849	0.00074
alvespimycin	-0.536	0.00098
8-azaguanine	-0.842	0.00107
ajmaline	0.918	0.00114
pioglitazone	0.547	0.00126
latamoxef	-0.904	0.00164
pivampicillin	-0.825	0.00179
butirosin	-0.821	0.00193
liothyronine	-0.82	0.00197
fluphenazine	-0.426	0.00223
sirolimus	-0.275	0.00237
flufenamic acid	-0.677	0.00304
meptazinol	-0.793	0.00368

P Values were calculated with multiple inspection correction for better credit (here we use Benjamini & Hochberg method for correction). And the corrected p Value is BH p. We chose BH p<0.001 as the Significant threshold for differentially expressed genes and found the expression of 13397 gene probes changed, which is linked to 10509 genes (See Supplement corcancer-cor.xls). These differentially expressed genes covered those genes co-function with B7-H3 and those changed after B7-H3 activity. Therefore, these differentially expressed genes can be helpful for clarify B7-H3 function mechanism.

GO Entries B7-H3 Participated in

Compared the GO clusters of differentially expressed genes of colon carcinoma to normal cells in Cellular component, Molecular function and Biological process, we obtained GO Entries as illustrated in Supplementary cellular component.txt, bioprocess.txt, molecular function. txt. Among which, B7-H3 participated in plasma membrane part (p=2.92xE-4). Obviously, B7-H3 functioned on the plasma membrane. Additionally, the Entry of lymphocyte activation also changed (p=4.85xE-7). Therefore, a critical change brought by B7-H3 alteration is the activation of lymphocyte, which is reported on the publications before.

Unearthing the Biological Pathways Associated B7-H3

To further unfold the mechanisms of B7-H3 function, we zoom in the biological pathways related. We chose the differentially expressed genes for KEGG sub pathway enrichment analysis. We got the changed signal pathways in colon carcinoma and selected the pathways associated with B7-H3. Here, we chose Benjamini<0.05, and at least two genes as the restricting condition for significantly changed biological pathways, illustrated in Table 1.

In the changed signal pathway, B7-H3 is located in Cell adhesion molecules (CAM). Furthermore, B7-H3 is reported to be a critical molecule in T cell immunity response reaction. Therefore, B7-H3 is directly related to T cell receptor signaling pathway. From these two signal pathways, we can find some clues of B7-H3 functions: when colon carcinoma occurs, B7-H3 is significantly high expressed as one of the components of cellular junction. Therefore, T cell receptor signal pathway is activated and hence performs the function of B7-H3.

Searching Small Molecules Mimic B7-H3 Function

As activation factor of T lymphocytes, B7-H3 can at some extent enhance body's immunity and hence perform immunity to cancer cells. With molecules mimic B7-H3 be found, we can enhance self-immunity ability and hence be helpful for colon carcinoma therapy.

Here we chose closely associated genes with B7-H3 (genes in the same GO Entry or pathway or whose expression changes in the same way in T cell receptor signaling pathway, see gene list at Supplementary CD276 RELATED GENE EXPRESSION.xlsx) to assess B7-H3 affections. These genes were classified into up-regulated and down-regulated and then compared to small molecules treated differential expression genes in CMAP database using GSEA to get some small molecules similar to B7-H3 functions. The most associated 20 molecules similar to B7-H3 functions are illustrated in Table 2 (the whole list of similarity between small molecules and B7-H3 is attached in Supplementary cmap.xls).

Therefore, small molecule ajmaline (enrichment=0.918) can imitate well as B7-H3, which means that ajmaline may enhance self-immunity ability in colon carcinoma therapy. When treating colon carcinoma, with ajmaline added as auxiliary, the therapy may improve.

Immunohistochemistry staining analysis of Colonic Cancer Cells and Normal Colonic Cells

There were brown coloring on the cell membrane of colonic cancer cells (Figure 2 A) and normal colonic cells (Figure 2 B). The expression of B7-H3 was observed in

Colonic Cancer Cells   Normal Colonic Cells
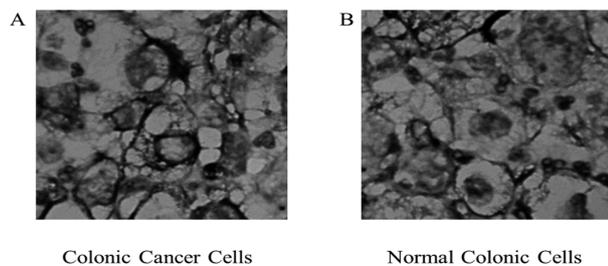
Figure 2. Immunohistochemistry Staining Analysis of Colonic Cancer Cells and Normal Colonic Cells (×400)

plasma-membrane of tumor cells. In the tumor tissues analyzed, immunocompetence was observed for B7-H3 of the 104 tumor tissues in both colonic cancer cells and normal colonic cells. This study indicates that the B7-H3 plays important roles in response to inmmuoreaction of colonic cancer cells in tumor proliferation and cell cycle arrest. H7-B3 is a favorable prognostic biomarker in the mechanism of colon carcinomas and provides the information regarding the likely targeted intervention.

Discussion

B7-H3 is newly discovered B7/CD28 superfamily member, functions as an important T cell immunity response molecule (Han et al., 2011). The fact that B7-H3 highly expressed in a lot of cancer cells induces the guess that B7-H3 is a regulation factor in automated tumor-resistant. Therefore, there is a deep and further meaning for B7-H3 mechanism research (Pourhoseingholi et al., 2010).

Seeing through the location of B7-H3 in GO clusters of differentially expressed genes, B7-H3 is associated with some other genes which changed in colon carcinoma plasma membrane, and these changes activated T lymphocyte. This point is supported by experiments before (Liu et al., 2011). The following pathway analysis also validated this point. Therefore, the possible pathway of B7-H3 functioning in colon carcinoma is the change of components on colon carcinoma plasma membrane and hence effect on the activation of T lymphocyte and then induce automatic immunity resistance (Sundarraj et al., 2010). This is also normal self-save method.

Through the GSEA methods, we found some small molecules mimic B7-H3 Function, such as tanespimycin, LY-294002, trichostatin A, ajmaline and so on. Heat shock protein 90 (HSP-90) is a kind of defense mechanism of cells, when cells encounter heat and other pressure (such as noxious cancer drugs), this mechanism will start. On tumor speaking, HSP-90 can help the cancer cells survive from death, if it doesn't work, the cancer cells are equivalent to suicide (Liu et al., 2011). HSP-90 molecules surround around malignant cells like lobster claw, and help cancer cells spread and growth, make the cancer cells to attack the human body. With this defense mechanism, the cancer cells can resist to the drug therapy. So many pharmaceutical companies have developed HSP-90 inhibitors to help the initial treatment exercise their "regular job". This inhibitor can be used to fight against all kinds of cancer (Vaishampayan et al., 2010), and ensure that tumor will not develop resistance to initial treatment drugs (Modi et al., 2007).

Also writing as LY-294002, LY-294002 or LY 294002, LY294002 is a common phosphatidylinositol 3-kinase (PI3K) inhibitors, can stop the acylation inositol protein kinase (Phosphotidylinsitol-3-kinase) in cell signaling pathways. LY294002 can pass through cells, and specifically inhibit PI3K, PI3K/Akt signaling pathways (Liu et al., 2010), including common inhibition of Akt phosphorylation (Zhong et al., 2010), etc. It is widely used in the study of PI3K cell signaling pathways (Du et al., 2010). As the derivant of quercetin, LY294002 is reversible, high-efficient PI3K kinase selective inhibitors, competitive combine to the ATP binding sites of enzyme. LY294002 has no inhibition on PI4K, DAGK, PKC, PKA, MAPK, S6K, EGFR and c-src tyrosine kinase. LY 294002 can inhibit in vitro proliferation of choroidal melanoma OCM-1 cells with IC50 = 10μM. When using colon cancer cells as the research object of in vivo and in vitro experiments, LY 294002 have shown both inhibition proliferation and induce the apoptosis of cancer cells. (Tang et al., 2009)

Notably, B cell receptor signaling pathway is found through pathway analysis to be changed. As we know, B lymphocyte, a critical member in automatic immunity, also changed in colon carcinoma cells. Therefore, B7-H3 may play certain role in this process. Only because this possible function unreported before, it is not included in the calculation on B7-H3 reaction. The automatic immunity pathway is activated, but the colon cancer cells remains. Therefore, we may conclude that in the colon carcinoma, certain step in the process from immunity activation to eliminate abnormal cell mutated and hence disarmed the automatic immunity function. Therefore, it seems very important to probe this step.

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