The Expression of Cancer Stem Cell Marker, CD133, and its Regulatory Mechanism in Digestive System

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The Expression of Cancer Stem Cell Marker, CD133, and its Regulatory Mechanism in Digestive System

Directed by Professor Mee-Yon Cho

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ABSTRACT

The Expression of Cancer Stem Cell Marker, CD133, and its Regulatory Mechanism in Digestive System

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Background: CD133 is a transmembrane glycoprotein that serves as a putative cancer stem cell (CSC) marker in various malignancies. CSCs have been described to have the capacity of progression, metastasis or resistance to chemo-radiotherapy in the malignant tumors. However, the undisputed role of CD133 expression and its regulatory mechanism in tumors of digestive tract is not clear yet. **Purpose of the study:** To understand the role of CSCs in tumors of digestive system, we

evaluated 1) the CD133 expression in various epithelial tumors as well as matched non-neoplastic mucosa of digestive system, 2) the regulatory mechanism of CD133 expression in colorectal cancer, 3) the prognostic significance of CD133 expression in colorectal cancer, and 4) the role of CD133 expression related to chemoresistence in colorectal cancer. **Methods:** The study includes 480 epithelial tumors of digestive system: 390 adenocarcinomas (271 CRC, 107 gastric (GC), and 12 pancreatic (PC)) and 90 gastroenteropancreatic neuroendocrine tumors (GEP-NETs) (15 gastric, 7 small intestine, 11 colon, 41 rectum, 4 gallbladder, 10 liver, and 2 pancreas) for clinical significance and three CRC cell lines (Caco-2, HT-29, and DLD-1) for *in vitro* test. Immunohistochemical (IHC) staining for CD133 was performed on the representative paraffin embedded blocks of surgically resected specimens in all 480 cases while real-time RT-PCR was performed on 75 CRC with available fresh frozen tissue. The level of promoter methylation was quantitatively analyzed by pyrosequencing on paraffin-embedded tissue in 171 CRCs. To evaluate the prognostic significance of CD133 expression in CRC and GEP-NETs, clinicopathologic data such as patients' age, sex, tumor location,

differentiation, invasion depth and lymph node metastasis were collected from the pathology reports. Patients who had undergone neo-adjuvant therapy were excluded. Survival data from national cancer registry were used for Kaplan-Meier survival analysis. In *in vitro* study, CD133 siRNA transfection was performed to Caco-2, HT-29, and DLD-1 cells and we subsequently evaluated the mRNA expression of genes related with chemoresistance (*ABCG2* and *AKT1*), cell proliferation and apoptosis (*β-catenin* and *Survivin*) with real-time RT-PCR. **Results:** In non-neoplastic mucosa of digestive tract, CD133 was expressed on the luminal side of cell membrane in few pyloric type glands in stomach, Brunner's glands in duodenum, small ducts and centroacinar cells in pancreas, bile ducts in liver and rare cells at crypt base in colon and rectum. Interestingly, CD133 was not expressed in non-neoplastic neuroendocrine cells of digestive tract including pancreatic islets. In neoplastic tissue, the pattern of CD133 expression was mostly luminal membranous and rarely dot-like cytoplasmic in adenocarcinomas in contrast to GEP-NETs that revealed mostly cytoplasmic (diffuse or focal) and rarely luminal expression. CD133 was positive in 35.7% of adenocarcinomas (48% of CRCs, 34% of

GCs and 25% of PCs) and 33% of GEP-NETs (30.3% of well-differentiated NET, 26.1% of poorly-differentiated neuroendocrine carcinomas, and 63.6% of mixed adenoneuroendocrine carcinomas). CD133 IHC expression was significantly correlated with CD133 mRNA expression level (P=0.0257). The methylation level of CD133 promoter was inversely correlated with CD133 IHC expression ($p<0.0001$) and CD133 mRNA expression level (P=0.11). However, no significant correlation between CD133 IHC expression in CRCs and clinicopathologic parameters such as age, sex, tumor histology, stage, invasion depth and lymph node metastasis was found. Similarly, CD133 expression in GEP-NETs was not correlated with tumor grade, site and expression of neuroendocrine markers (chromogranin-A and synaptophysin). Survival analysis in stage II & III CRC patients revealed no significant correlation between CD133 expression and overall survival (OS) (P=0.9689) or disease-free survival (DFS) (P=0.2103). Importantly, CD133+ tumors were significantly associated with longer OS in patients with adjuvant therapy compared to those without adjuvant therapy ($p<0.0001$, HR 0.125, 95% CI 0.052-0.299). However, patients with CD133- tumors did not show significant

difference in survival according to adjuvant therapy (P=0.055, HR 0.500, 95% CI 0.247-1.015). CD133 was also not correlated with OS in neuroendocrine carcinoma patients (P=0.97). In multivariate analysis, CD133 was not an independent prognostic factor in CRC. CD133 was expressed in all three CRC cell lines as evidenced by RT-PCR. The results of qRT-PCR in CRC cell line after CD 133 siRNA transfection indicated that the expression of *ABCG2* and *AKT1* mRNA was increased while that of *β-catenin* and *Survivin* was decreased at 48 and 72 hour. **Conclusions:** CD133 has a distinctive expression pattern and distribution in adenocarcinomas as well as neuroendocrine neoplasms of digestive system in contrast to its focal or rare expression in non-neoplastic mucosa. CD133 expression seems to be negatively regulated by methylation of its promoter in CRCs. Importantly, this is the first report of CD133 expression in GEP-NETs showing lack of expression in non-neoplastic neuroendocrine cells, indicating an impelling finding that needs further clarification. Although CD133 expression is not an independent prognostic marker, it may prove helpful in predicting the benefit of adjuvant therapy in stage II & III CRCs. This hypothesis was further supported by the in vitro study

which showed an increase in the mRNA expression of genes related to chemoresistance (*ABCG2* and *AKT1*) after CD133 knock-down by siRNA transfection in colon cancer cell lines.

Key words: Cancer stem cell, CD133 protein, human, digestive system neoplasms, adenocarcinoma, neuroendocrine tumor, chemoradiotherapy, adjuvant, prognosis

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I. INTRODUCTION

Cancer is an abnormal growth in a tissue that owns the capacity of unlimited growth and becomes resistant to various survival stresses and harmful stimuli. Accumulating experimental evidences indicate that cancer stem cells (CSC) are responsible for the initiation and progression of cancer. This idea challenges the classic stochastic model of cancer development. Cancer cells with features of stem cells were first described by Rudolf Virchow in the mid 19th century while

he observed that the histological characteristics, proliferation and the capacity of differentiation of some cancer cells are similar to embryonic stem cells.[2] After the invention of flow cytometry, the isolation of subsets of cells were made feasible through the use of specific markers.[3] The first experimental evidence of cancer stem cells emerged in 1997 when Bonnet et al isolated two groups of leukemic cells using CD34 and CD38 (CD34+CD38+ and CD34+CD38-) both of which revealed different capacity of self-renewal, multi-differentiation and the speed with which the resulting tumors grew.[4] It was followed by the isolation of CD44+CD24- cancer stem cells in breast cancer in 2003.[5]

CSCs are undifferentiated cells that expand their colony through asymmetric cell division, the result of which is two daughter cell population, one being similar to the mother cells, retaining stem cell properties, while the other one is committed to undergo a specified differentiation.[6] CSCs have been isolated from many hematologic and solid tumors including colorectal (CRC), gastric (GC) and pancreatic cancers (PC)s and they have been defined to have the capacity of self-renewal and multipotency[7] and ability to maintain the stem cell pool

and most elements of the tumor for unlimited time period[8-9] being responsible for tumor initiation and progression[10], resistance against chemo-radiotherapy, and relapse after initial eradication.[11]

Where the cancer stem cells originate from is still unknown, however the potential origin of cancer initiating cells are thought to be the adult stem cells, embryonic stem cells, progenitors with unlimited self-renewal capacity, and/or induced pluripotent stem cells.[12]

Different markers have been found to be expressed on the surface of CSCs, out of which CD133 has retained much attention and importance.

The CD133+ population exists among cancer initiating cells in many tissues, including carcinoma of colon[13], breast[14], lung[15], stomach[16], liver[17], gallbladder[18], prostate[19], endometrium[20], and pancreas[21], leukemia[22], glioma[23], and medulloblastoma.[24]

CD133 or Prominin-1 is a pentaspan transmembrane glycoprotein (Figure 1), whose gene is located on chromosome 4p15.32.[25] CD133 comprises five transmembrane domains and two large glycosylated extracellular loops.[26]

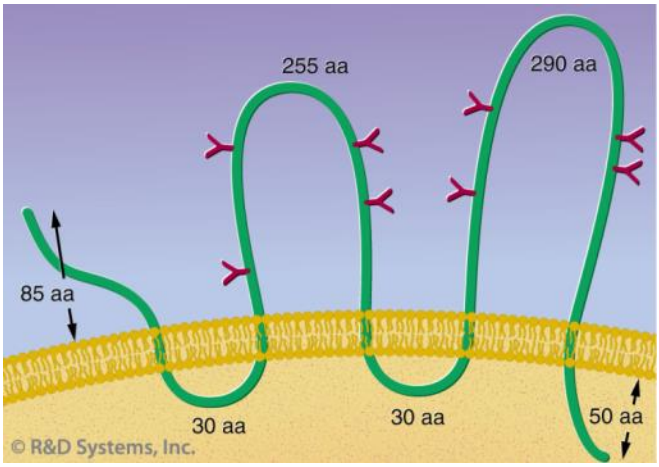

Figure 1. Structure model of CD133 glycoprotein. CD133 is a pentaspan transmembrane glycoprotein with an extracellular N-terminus, 5 hydrophobic transmembrane domains, 2 small cytoplasmic loops, 2 large extracellular loops and a cytoplasmic C-terminus. (Adopted[27])

Three of the five promoters responsible for CD133 transcription are located in a CpG island.[28] Thus, epigenetic factors can complicate the regulation of CD133 gene transcription.[29] DNA hypomethylation is accounted as an important determinant of CD133 expression.[30] Shmelkov et al further stated that the transcript of CD133 is found in many cell lines and most adult tissue. However, the AC133 antigen of CD133 (a glycosylated form of CD133) has been documented to be more restricted to undifferentiated cells, including endothelial progenitor cells[31], hematopoietic stem cells[32], fetal brain stem cells[33], embryonic epithelium[34] and certain cancers.

Transforming growth factor beta 1 (TGFb1), a multifunctional cytokine was demonstrated to be able to regulate CD133 expression through inhibition of DNMT1 (DNA methyl transferase) 1, and DNMT3b expression and subsequent demethylation of promoter-1 of CD133 gene[35] (Figure 2).

However, yet the regulatory mechanism of CD133 gene transcription is not utterly understood.

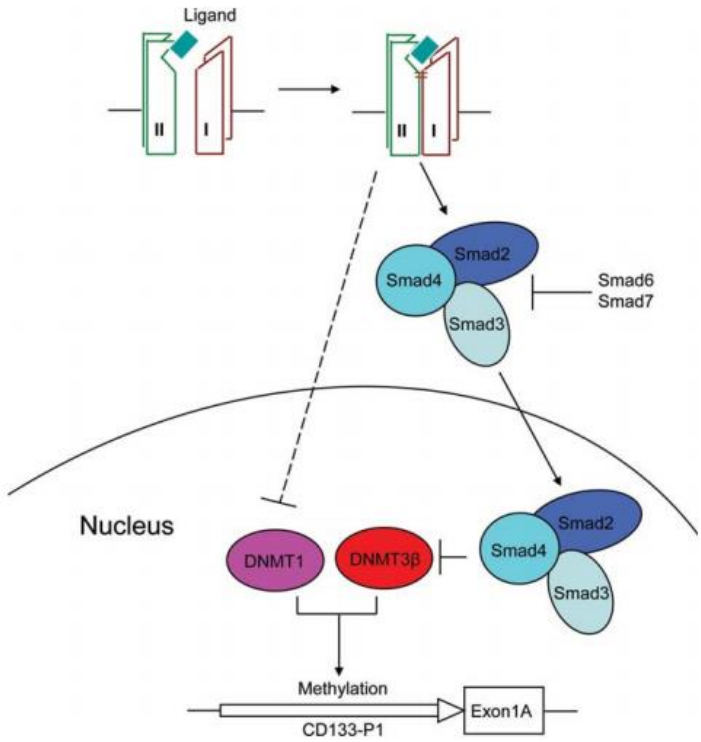

Figure 2. The proposed scheme by which TGFb1 regulates CD133 expression. By binding of TGFb to its receptor, Smad heterocomplex will be formed and this complex will suppress the expression of DNA methyl transferases (DNMTs). As a result of lower levels of DNMTs, hypomethylation of CD133 promoter-1 occurs which results in increased CD133 transcription. (adopted[35])

CD133 expression is reported to be indicative of a resistance phenotype[36], poor prognosis[37], and is believed to mediate cancer relapse after chemotherapy[38] and lower level of CD133 mRNA expression is documented to be associated with a longer relapse-free interval and overall survival (OS) in cancers such as colorectal.[39]

Controversially, it was recently shown that CD133(+) cells are not more resistant to chemotherapy than CD133(-) cells.[40] On the other hand, the evidence provided by Huang, E. H. et al shows that nude mice injected with CD133(-) colon cancer cells developed cancer.[41] Moreover, Du. L. et al have demonstrated that knock-down of CD133 does not compromise the tumor-initiating capability of colon cancer cells, questioning a functional role of this molecule for the colon cancer stem cells.[42]

Although the existence of CSCs in adenocarcinomas have been previously described, proper evaluation of CD133 IHC expression and its prognostic significance is needed because previous data in literature come from studies that have superficially assessed CD133 expression. For example, the numbers of patients in the studies were either too few

or they used tissue microarray for immunohistochemistry which due to the heterogeneous nature of CD133 expression cannot properly represent the real value. Additionally, the use of different cutoff values for the positivity of CD133 and counting CD133 positive cells only in hot-spot areas, lack of evaluation of effective factors such as tumor stage or adjuvant therapy are other reasons for the previous contradictory results of CD133.

Different CSC markers have been identified which are organ-specific. However, there is no mention about the correlation of CSC markers and histologic types of tumors in the same organ. Epithelial malignant tumors of digestive system are mainly divided into carcinoma (adenocarcinoma, adenosquamous carcinoma, carcinoma with lymphoid stroma, hepatoid adenocarcinoma, and squamous cell carcinoma) and neuroendocrine neoplasms. The most common malignant tumor among them is adenocarcinoma in every organ of digestive tract. Neuroendocrine neoplasms are rare but are seen in every organ of digestive tract because they arise from the cells of diffuse neuroendocrine system of the digestive tract.

However, the existence of CSCs in gastroenteropancreatic neuroendocrine tumors (GEP-NETs) remains largely unexplored.

GEP-NETs are rare with an incidence of 1 per 100,000 population for pancreatic and 1.95-2.5 per 100,000 for gastrointestinal tumors.[43-44] However, the incidence has substantially increased in different parts of the world over the last three decades thanks partly to the increased availability of advanced endoscopic and radiologic imaging.[45-46]

GEP-NETs arise at every location where endocrine precursor cells are located and comprise tumors with variable biologic behavior depending on the characteristic of the cells from which that the tumor originates. They were primarily classified according to the site of origin as foregut (respiratory system, stomach, duodenum, proximal jejunum and pancreas), midgut (distal jejunum, ileum, appendix and right hemicolon) and hindgut (left hemi-colon and rectum).[47]

The term carcinoid was initially used for all neuroendocrine tumors assuming their uncertain potential of malignancy, but is now replaced by the term neuroendocrine neoplasms (NENs).

Recently NENs are further divided into three main categories: well-differentiated neuroendocrine tumor (NET), poorly differentiated neuroendocrine carcinoma (NEC), and mixed adenoneuroendocrine carcinoma (MANEC) (Table 1).[46] There is no evidence so far to indicate the transformation or progression of a well-differentiated NET to a NEC and neither there has been any report to show the occurrence of metastatic NEC from a well-differentiated NET. MANEC has a phenotype that is morphologically recognizable as both gland-forming epithelial and neuroendocrine, with both components being malignant and each component comprises at least 30% of the tumor.

MANECs raise the concept of a "histogenetic tumor typing". Molecular analysis of MANECs defines that different components in these tumors have a common clonal origin.[48] Moreover, it is believed that the tumors of the gastrointestinal tract arise from a common stem cell.[49]

Table 1. WHO Classification of NENs (2010)

NET G1	Mitotic Count <2/10 HPF Ki-67 index ≤2%
NET G2	Mitotic Count 2-20/10 HPF Ki-67 index 3-20%
NEC* (large or small cell type)	Mitotic Count >20/10 HPF Ki-67 index >20%
MANEC**	Adenocarcinoma and Neuroendocrine carcinoma

HPF; high power field

*Also referred to as NET G3

**Both components should be malignant, each comprising at least 30% of tumor

All NENs are potentially malignant in contrast to the belief held when it was first diagnosed.[50] The clinical course of NETs is often indolent but resistant to chemotherapy.[51] Additionally, patients with NECs are at increased risk of developing synchronous or metachronous non-endocrine malignancies.[52]

Recently, the existence of small population (5.8% ± 1.4%) of putative CSCs was demonstrated in the NETs of gastrointestinal tract using Aldefluor assay from 19 primary patients' samples.[53] They also showed that when the neuroendocrine cell line, CNDT96 was positive for ALDH (a putative CSC marker), it revealed higher tumor growth and sphere forming ability. However, these cells failed to show immunoreactivity for CD133. On the other hand, lung small cell carcinoma (NEC) cell lines were reported to express CSC marker, CD133, and these cells revealed stem cell-like features, including self-renewal, differentiation, proliferation and chemoresistence.[54-55]

However, the expression of CD133 in clinical samples of GEP-NETs has not been reported yet.

CSCs are attributed with their ability to confer resistance to chemoradiotherapy and escape various toxic stimuli and apoptosis caused by therapy. Chemoresistence of CSCs has been related to some of following; expression of ATP binding cassette (ABC) transporters such as MDR1 and *ABCG2*, high expression of aldehyde dehydrogenase (ALDH), expression of anti-apoptotic proteins such as *Survivin* or pro-survival B-cell lymphoma 2 (BCL-2) protein family members and CSC-related pathways such as *Wnt/β-catenin*, *MYC*, and *AKT1*.[56] The correlations between CD133 expression as a CSC marker and the above-mentioned factors that are related to chemoresistence have not been clearly elucidated.

In this study, we evaluated a) the pattern and distribution of CD133 expression by immunohistochemical (IHC) stains in non-neoplastic mucosa of digestive tract and neoplastic tissue (colorectal, gastric and pancreatic adenocarcinomas and GEP-NETs), and validated the IHC results with real-time RT-PCR, b) the role of methylation of CD133 promoter in the regulation of CD133 expression, and c) the prognostic significance of CD133 expression in CRCs and GEP-NETs. Additionally, using colorectal cancer cell lines, we evaluated the effect

of CD133 inhibition by siRNA transfection on the mRNA expression level of genes related to chemoresistence (*ABCG2* and *AKT1*), apoptosis (*Survivin*) and cell proliferation (*β-catenin*).

II. MATERIALS AND METHODS

1. Materials

A. Clinical Samples

Three hundred ninety patients with adenocarcinomas and 90 patients with neuroendocrine neoplasms of digestive tract were included in this study. Patients with adenocarcinoma included 271 colorectal, 107 gastric and 12 pancreatic cancers that were surgically resected and pathologically diagnosed at Wonju Severence Christian Hospital from 2000 to 2006. Patients with GEP-NETs were 90 in number and were from different location (stomach, n=15, duodenum, n=6, ileum, n=1, appendix, n=3, colon, n=8, rectum, n=41, pancreas, n=2, gallbladder, n=4 and liver, n=10).

Paraffin-embedded sections from all cases included in the study were used for IHC staining of CD133.

For evaluating the regulatory mechanism of CD133 expression, we used the fresh frozen tissue (n=75) from colon and rectum for real-time

RT-PCR and paraffin-embedded sections (n=171) from colon and rectum for methylation analysis using pyrosequencing.

B. Colorectal Cancer Cell Lines

Three colorectal cancer cell lines (Caco-2, HT-29, and DLD-1) that were previously shown to express high level of CD133 were used for *in vitro* study.

2. Ethics Approval

The study has been approved by the Institutional Ethic Committee of Yonsei University, Wonju College of Medicine (YWMR-12-4-031) and has been carried out in compliance with the guidelines of the Declaration of Helsinki.

3. Histologic Evaluation of GEP-NET

All slides of GEP-NETs included in this study (n=90) were re-evaluated and graded based on WHO 2010 classification for NENs of the digestive system: NET G1, NET G2, NEC and MANEC.[57] The grading of GEP-NETs was based on the assessment of proliferation fraction and mitosis[58] (Table 1 & Figure 3).

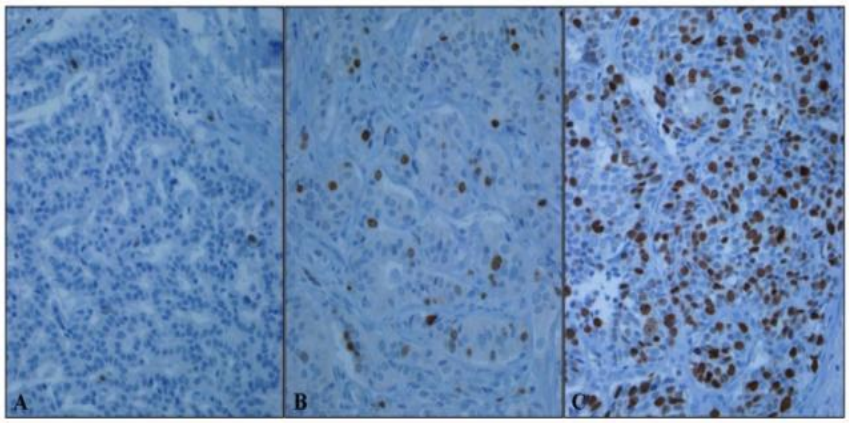

Figure 3. Ki-67 labeling index in NENs. The expression of Ki-67 in NENs shows the proliferative index of the tumor (A) NET G1 (0-2%), (B) NET G2 (3-20%) and (C) NEC or NET G3 (>20%).

The growth pattern of tumor cells at microscopic level was evaluated according to Soga classification. Based on that, GEP-NETs are divided into five categories according to the growth pattern of the neuroendocrine tumor cells. Type I refers to the pattern in which tumor cells are arranged in solid, nodular or insular pattern while in type II, tumor cells are arranged in trabecular pattern or in ribbons with frequent anastomosing growth. In type III, tumor cells make tubules, acini and glands or rosette-like pattern and in type IV, tumor cells show atypical growth and features of poor differentiation, while in type V, a combination of the above-mentioned growth patterns are observed[59] (Figure 4).

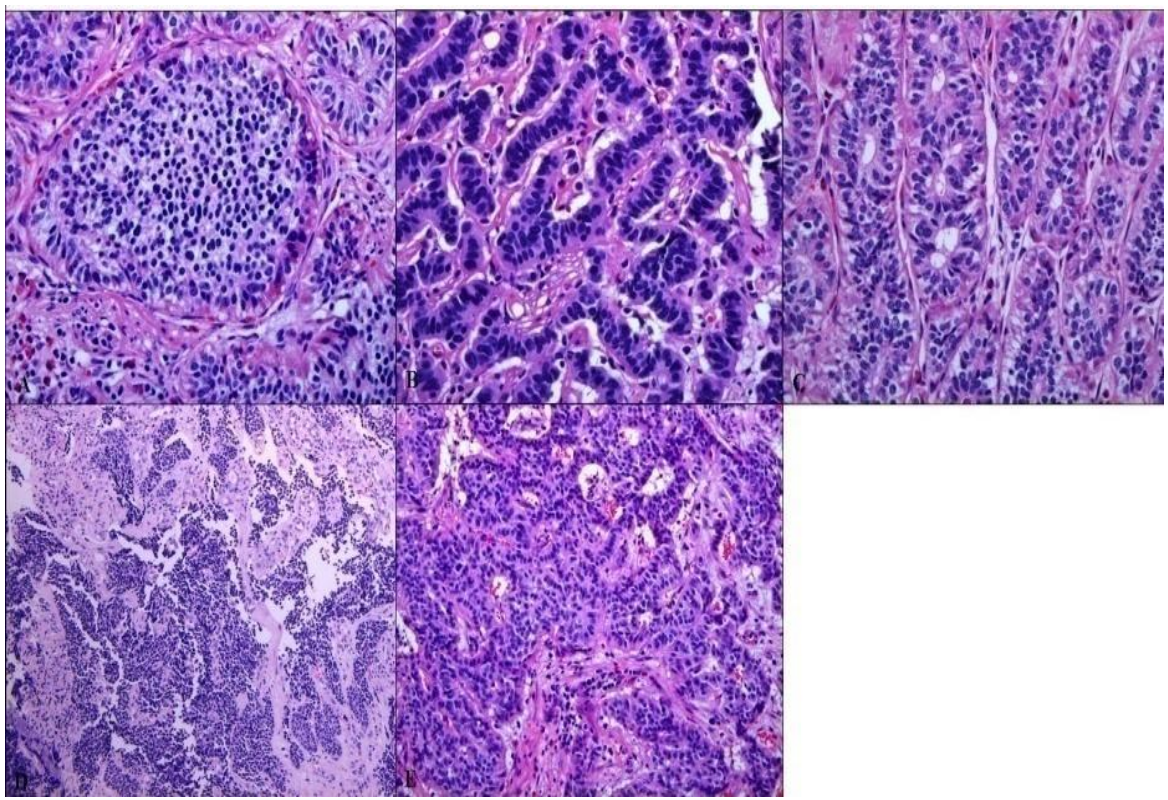

Figure 4. The growth patterns of neuroendocrine tumors. Neuroendocrine tumor cells grow in different patterns, (A) nodular, (B) trabecular, (C) acinar, (D) poor differentiation, and (E) mixed.

4. Collecting Data for the Prognostic Analysis of CD133 Expression in CRC and GEP-NETs

The prognostic significance of CD133 was evaluated only in colorectal cancer patients and GEP-NETs. For the evaluation of prognostic significance of CD133 expression in CRCs and GEP-NETs, the data of clinicopathologic parameters of patients including age, gender, tumor location, invasion depth, histologic differentiation, and lymph node metastasis were collected from the pathology report. Survival data from national cancer registry were used for Kaplan-Meier survival analysis.

Patients with colorectal adenocarcinoma had surgically resected stage II and III CRC and available follow-up information. For the comparison, we divided the patients according to the tumor location and either receiving adjuvant treatment or no adjuvant treatment. The patients receiving preoperative chemotherapy or radiotherapy were excluded. Survival data from national cancer registry were used for Kaplan-Meier survival analysis.

Colorectal cancer patients were planned to receive 5th cycles to 12 cycles of adjuvant FOLFOX chemotherapy within a six-month period. The patients received a 2-hour infusion of 85 mg of oxaliplatin per square meter on day 1, in addition to the standard LV5FU2 regimen (FOLFOX4) or the simplified LV5FU2 regimen (modified FOLFOX6). After surgery, tumor recurrence was detected by physical examination, serum CEA antigen assay, and abdominal imaging every three to six months for 3 years, every six months for the following 2 years, and then annually. The duration of follow-up was defined as the time between surgery and disease recurrence (DFS), death (OS), or last hospital contact (scheduled follow-up or telephone contact). Maximum follow-up period was 108.3 months.

Survival analysis was also performed in 23 patients with NECs who had available follow-up data. The tumor locations in these patients were as following; stomach (n=5), duodenum (n=2), pancreas (n=1), gallbladder (n=2), liver (n=10), colon (n=1) and rectum (n=2). Meanwhile, the T-stage of the tumor were as following; T1 (n=3), T2 (n=4), T3 (n=5), T4 (n=1), and metastatic (n=10).

5. Immunohistochemical Analysis of CD133 in Clinical Samples

Paraffin-embedded tissue sections from representative blocks were deparaffinized with xylene and then rehydrated through graded alcohol solutions. Antigen retrieval (according to manufacturer's instructions) consisted of slide warm-up to 75°C and incubation for 4 minutes followed by applying cell conditioning solution #2 for 60 minutes (Ventana medical system, Roche, Tucson, USA). Endogenous peroxidase was blocked by applying UV inhibitor for 4 minutes. The slides were then washed with reaction buffer. The Ultra View Universal DAB Detection Kit was used for IHC staining. The steps are briefly described as following. The primary antibody (CD133/1 (AC133) pure, Human, MACS, Miltenyi Biotec, CA, USA) was applied and incubated for 2 hours at a 1:100 dilution in Ventana machine (BenchmarkXT, Ventana medical system, Roche, Tucson, USA). Slides were then rinsed with reaction buffer and added one drop of HRP UNIV MULT, DAB and DAB H2O2 (Ventana medical system, Roche, Tucson, USA) for 8 minutes each, consecutively with

intermittent rinsing with reaction buffer.

Slides were then treated with one drop of COPPER for 4 minutes before counterstaining with hematoxylin for 4 minutes, followed by bluing agent and finally rinsed with reaction buffer.

The IHC stain for neuroendocrine markers, chromogranin-A (DAKO, Glostrup, Denmark), synaptophysin (Ventana, Roche, Tucson, USA), and Ki-67 (Thermo Fisher Scientific, CA, USA) was done with same method mentioned above except for using cell conditioning solution #1 instead of #2.

6. Interpretation of CD133 Immunostaining

The IHC staining for CD133 was scored as 0 when there was no expression at all, 1+ when the expression of CD133 was detected in 1-10% of the whole tumor area, 2+ when it was expressed in 11-50% of tumor area and 3+ when it was expressed in 51-100% of the tumor area. Tumors with CD133 expression on over 10% of whole tumor area were considered as CD133 positive (Table 2). We used the normal proximal tubules of kidney as positive control for CD133 (Figure 5).

The IHC staining results were evaluated independently by two pathologists blinded to the patients' clinical and pathologic information. Discrepancies between the pathologists were resolved by consensus.

Table 2. The evaluation of CD133 IHC staining and its interpretation

Groups	CD133+ area in the tumor	Interpretation
0	0%	Negative
1	1-10%	Negative
2	11-50%	Positive
3	51-100%	Positive

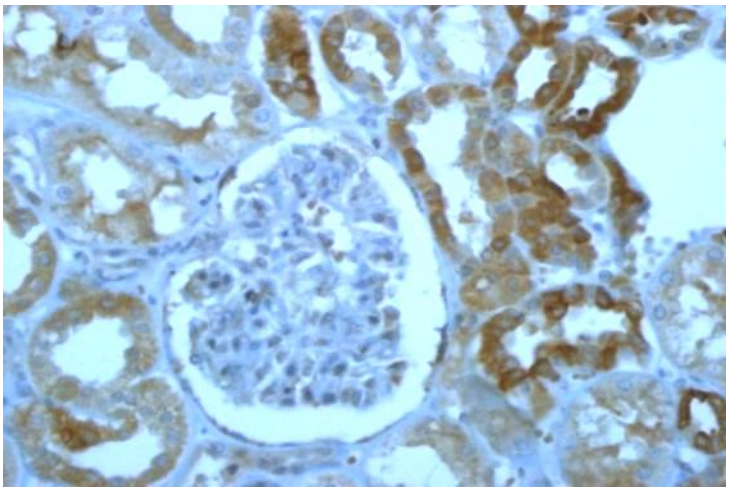

Figure 5. Positive control for CD133 immunohistochemical stain. The proximal tubules of normal kidney were used as positive control.

7. RNA Extraction and cDNA Synthesis of CD133 in CRCs

Fresh frozen tissues after surgery were available in 75 out of 271 cases of CRC. The total RNA was extracted from 20mg colorectal frozen tissue, using RNeasy plus Mini kit (QIAGEN Hilden, Germany) according to manufacturer's protocol and Quantitect Reverse Transcription kit (QIAGEN Hilden, Germany) was used for cDNA synthesis from 500ng of total RNA.

8. Quantitative RT-PCR for CD133 in CRCs

Real-time RT-PCR was performed (as described elsewhere[1]) in 384 well PCR plates containing the Fast SYBR Green Master Mix (Applied Biosystems, California, USA), cDNA template, CD133 RT sense primer (5'-CTGGGGCTGCTGT TTATTATTCTG-3'), and CD133 RT antisense primer (5'-ACGCCTTGTCCTTGGTAGTGTTG-3') in a final volume of 10 μL. Each primer/cDNA set was set up in triplicate. Real-time PCR reactions in a 7900HT Fast Real-Time PCR System (Applied Biosystems) were initiated by heating to 50°C for 2 min and then to 95°C for 10 min, followed by 40 cycles of 95°C (15 s),

and 60°C (60 s). The relative quantification of gene expression was performed using the ΔΔCt method.

9. Bisulfite Conversion and Pyrosequencing Analysis of DNA Methylation of CD133 Promoter in CRCs

We extracted DNA from microdissected samples using DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Genomic DNA was modified with sodium bisulfite using an EpiTect® Bisulfite kit (QIAGEN Hilden, Germany) according to manufacturer's instruction. Methylation status of CD133 was assessed using pyrosequencing-based methylation analysis. We evaluated the methylation status of CpG sites in promoter P2 and exon 1B, as these sites have previously shown correlation with CD133 gene transcript[1] (Figure 6). All primers for pyrosequencing were designed with Pyrosequencing Assay Design (QIAGEN, Hilden, Germany). Bisulfite-treated genomic DNA was used as a template in subsequent polymerase chain reactions. For each gene, a 30ul PCR was carried out with HotStarTaqPlus Master Mix (QIAGENE, Hilden, Germany) to label bisulfite converted DNA with biotinylated primers (CD133 promoter site: Forward 5'-GGAGTAGGGATATGGGGGTATAAA-3', Reverse primer 5'- AAACACCCCAATTCTCCATCT-3'). The

PCR conditions included denaturation 94°C (30s), annealing 54°C (30s), and extension 72°C (30s) and 40 cycle. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers (5'- GGGATATGGGGGTATAAA-3'). The sequencing primer includes four methylation sites (GYGAGGTTATTTTTTYGYGTTYGTGGG). Pyrosequencing was performed with PSQ HS 24 Gold single-nucleotide polymorphism reagents on a PSQ HS 24 pyrosequencing machine (Biotage, Uppsala, Sweden).

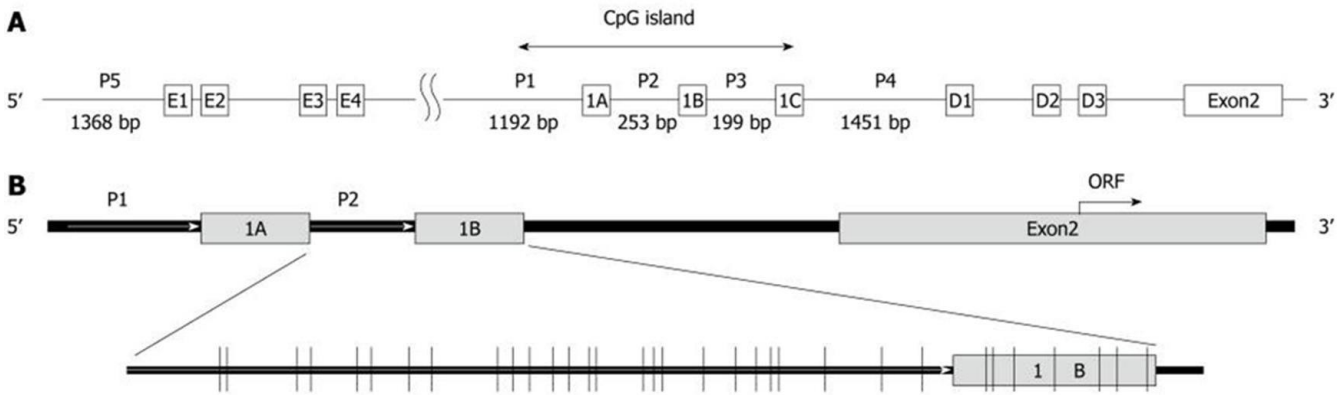

Figure 6. The promoter profile of CD133. CD133 has five promoters three of which are located at a CpG island (A). We set our sequencing primer at P2 and Exon 1B (B) (Adopted [1]).

10. Inhibition of CD133 Expression by siRNA Transfection in CRC Cell Lines

Caco2 cells were maintained in MEM/EBSS while HT-29 and DLD-1 cells were maintained in RPMI-1640 with 10% fetal bovine serum. 10 nM and 20 nM siRNAs (QIAGEN, Maryland, USA) targeted against four different parts of the CD133 gene sequence (Table 3) was mixed with 200 µl PBS and 4 µl G-fectin (Genolution, Seoul, Korea, Cat No. 1027416) and incubated for 10 min at room temperature. Approximately 8 x 10^4 DLD-1 cells were plated in the six-cluster plates followed by treating them with the above incubated transfection mixture. Cells were harvested at 48 and 72 hour post-transfection. Subsequently, total RNA was extracted from colorectal cancer cell lines (Caco-2, HT-29, DLD-1), using RNeasy plus Mini kit (QIAGEN Hilden, Germany) according to manufacturer's protocol and HotstarTaq Plus master Mix kit (QIAGEN, Hilden, Germany) was used for cDNA synthesis from 1µg of total RNA.

Table 3. List of target sequences of CD133 gene for which siRNA was targeted

Product name	Catalog no.	Target sequence
Hs_PROM_2	SI00083741	CACGTTATAGTCCATGGTCCA
Hs_PROM_3	SI00083748	CAGGTAAGAACCCGGATCAAA
Hs_PROM_4	SI00083755	ACCTTTGAGTTTGGTCCCTAA
Hs_PROM_5	SI03098263	CTGGCTAAGTACTATCGTCGA

11. Quantitative RT-PCR for CD133, *ABCG2*, *AKT1*, *Survivin* and *β-catenin* in CRC Cell Lines

Real-time RT-PCR was performed as described elsewhere[1] in 384 well PCR plates containing the Fast SYBR Green Master Mix (Applied Biosystems, California, USA), cDNA template, CD133 RT sense primer (5'- CTGGGGCTGCTGTTTATTATTCTG-3'), and CD133 RT antisense primer (5'-ACGCCTTGTCCTTGGTAGTGTTG-3') or *ABCG2* RT sense primer (5'-CTTGGATGAGCCTACAACTGGC-3'), and ABCG2 antisense primer (5'-GTTCGTCCCTGCTTAGACATCCT-3'), *Survivin* RT sense primer (5'-GGCAGCCCTTTCTCAAGGAC-3'), and *Survivin* antisense primer (5'-CCAAGTCTGGCTCGTTCTCAGT-3'), *β-catenin* RT sense primer (5'-TCGCCTTCACTATGGACTACCAG-3') and *β-catenin* antisense primer (5'-GTGCACGAACAAGCAACTGAAC-3') and *AKT1* sense primer (5'-CATCACACCACCTGACCAAGAT-3') and *AKT1* antisense primer (5'-TGGCCGAGTAGGAGAACTGG-3') in a final volume of 10 pmole each. Each primer/cDNA set was set up in triplicate. Real-time PCR reactions in a 7900HT Fast Real-Time PCR System (Applied

Biosystems) were initiated by heating to 50°C for 2 min and then to 95°C for 10 min, followed by 40 cycles of 95°C (15 s), and 60°C (60 s). The relative quantification of gene expression was performed using the ΔΔCt method.

12. Statistical analysis

χ^2-test and Mantel-Haenszel test were used to analyze the categorical data. We used Pearson correlation to compare distributions of qualitative variables. Survival curve was estimated with the Kaplan-Meier method and compared using the log-rank test. Multivariate Cox proportional hazard regression model was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) with adjustment for age and stage. Analyses were performed using SAS (Version 9.2, SAS Inc., North Carolina, USA). A value of $p<0.05$ was considered statistically significant.

III. RESULTS

1. Immunohistochemical Expression of CD133 in Clinical Samples

A. Pattern and Distribution of CD133 Expression in Non-neoplastic Mucosa of Digestive Tract

The CD133 expression in non-neoplastic mucosa of digestive system (wherever observed) was exclusively localized to the luminal side of the cell membrane. CD133 was not expressed in the cytoplasm of the non-neoplastic mucosa or parenchyma in any of the cases. CD133 expression was not observed in the non-neoplastic neuroendocrine cells that are scattered throughout the gastrointestinal tract.

In stomach, there was focal CD133 expression along the luminal side of some non-endocrine cells of normal pyloric glands (Figure 7A). There was no immunohistochemical expression of CD133 either in cardiac or body type glands or in glands with intestinal metaplasia (Figure 7B).

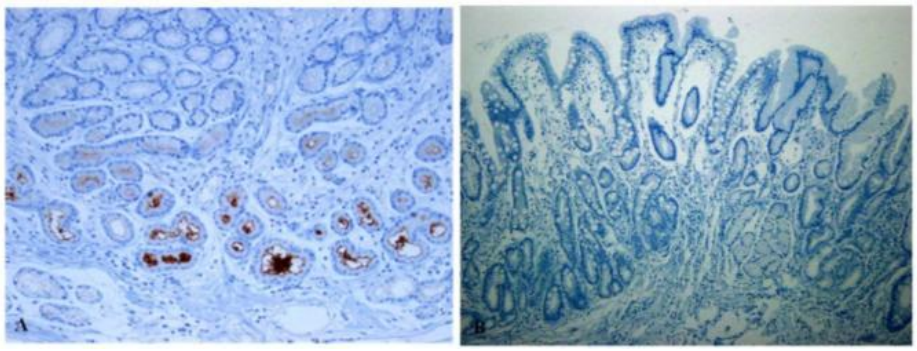

Figure 7. The immunohistochemical expression of CD133 in non-neoplastic mucosa of stomach. Rarely, CD133 was expressed along the apical side of pyloric type glands (A). CD133 was negative in non-neoplastic gastric mucosa that showed intestinal metaplasia (B).

The non-neoplastic mucosa of duodenum showed CD133 positivity along the luminal side of membrane in Brunner's glands in some cases (Figure 8A). The mucosal lining of the duodenum was devoid of CD133 immunoreactivity. There was rare positivity for CD133 along the apical side of cell membrane in the base of jejunum crypts (Figure 8B).

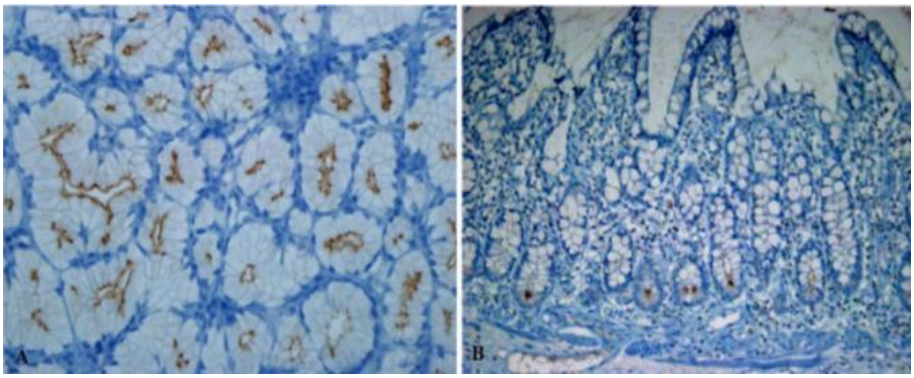

Figure 8. The immunohistochemical expression of CD133 in non-neoplastic mucosa of small intestine. There was seldom apical positivity in Brunner's glands (A) and rare few cells positive in crypt base in jejunum (B).

No detectable unequivocal CD133 immunoreactivity was observed in non-neoplastic mucosa of appendix and gallbladder. However, a weak CD133 IHC expression in non-neoplastic colorectal mucosa around the tumor was noted in a few scattered cells and luminal border at the base of normal crypts (Figure 9).

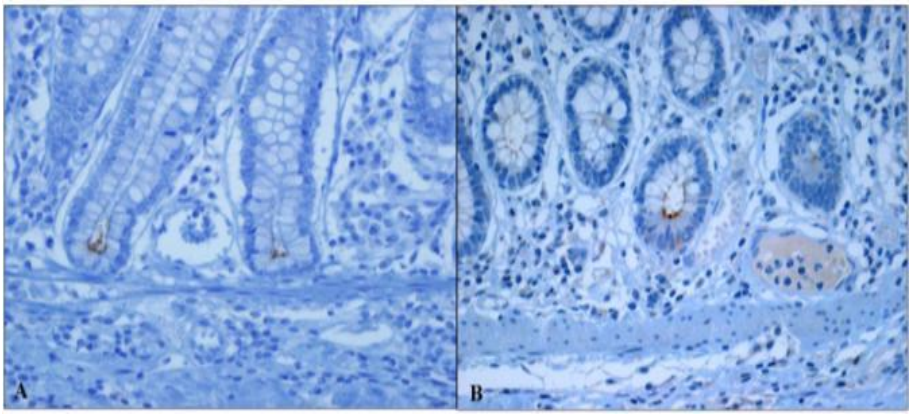

Figure 9. The immunohistochemical expression of CD133 in non-neoplastic mucosa of large intestine. Rare CD133 positive cells were observed in the crypt base in non-neoplastic mucosa colon and rectum (A&B).

The non-neoplastic pancreatic islets which are aggregations of endocrine cells were exclusively non-reactive for CD133 (Figure 10A). The lining epithelia of large to medium-sized pancreatic ducts were also devoid of CD133 expression. However, the lining epithelia of small ducts, ductules and centroacinar cells showed positivity for CD133 along the luminal side of cell membrane.

In the non-neoplastic liver, CD133 was expressed in few small bile ducts and ductules (Figure 10B). There was no expression of CD133 in the hepatocytes and large bile ducts.

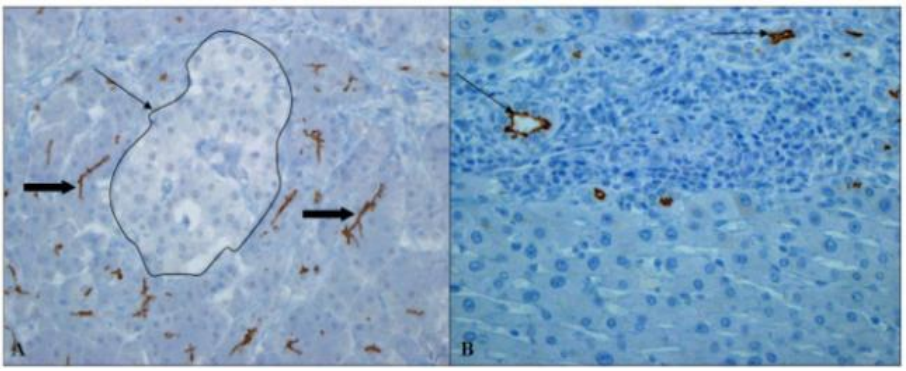

Figure 10. CD133 immunohistochemical expression in non-neoplastic pancreas and liver. In non-neoplastic pancreas (A), luminal pattern of CD133 expression is observed in small pancreatic ducts and centroacinar cells (thick arrows), however, there is no immunoreactivity for CD133 in the pancreatic islets (thin arrow points to the pancreatic islet which is encircled by a imaginary dentate line and appears clearer than surrounding exocrine parenchyma). In non-neoplastic liver (B), CD133 immunohistochemical expression is observed along the luminal side of the apical membrane in small bile ducts and ductules (arrows), while hepatocytes are exclusively devoid of CD133 expression.

B. The Pattern of CD133 IHC Expression in Tumors of Digestive Tract

(1) Adenocarcinomas

In adenocarcinomas of either organ (colorectal, gastric and pancreatic), CD133 IHC expression was seen exclusively on the cell membrane at the glandular luminal surface of cancer glands (Figure 11A). Rarely, tumors with poor differentiation with no gland formation (Figure 11B) showed dot-like cytoplasmic expression for CD133.

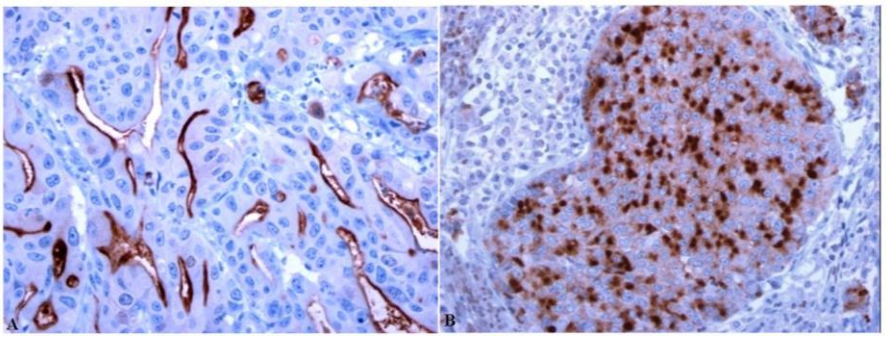

Figure 11. CD133 immunohistochemical expression patterns in adenocarcinomas. CD133 was mostly expressed at the luminal side (A) and rarely in the cytoplasm (dot-like, B).

The intraglandular debris of shed tumor cells in some cases showed CD133 immunoreactivity, which were not taken into account.

The CD133 IHC expression was observed in tumors with different differentiation (well, moderate and poor differentiation) as well as mucinous adenocarcinomas (Figure 12).

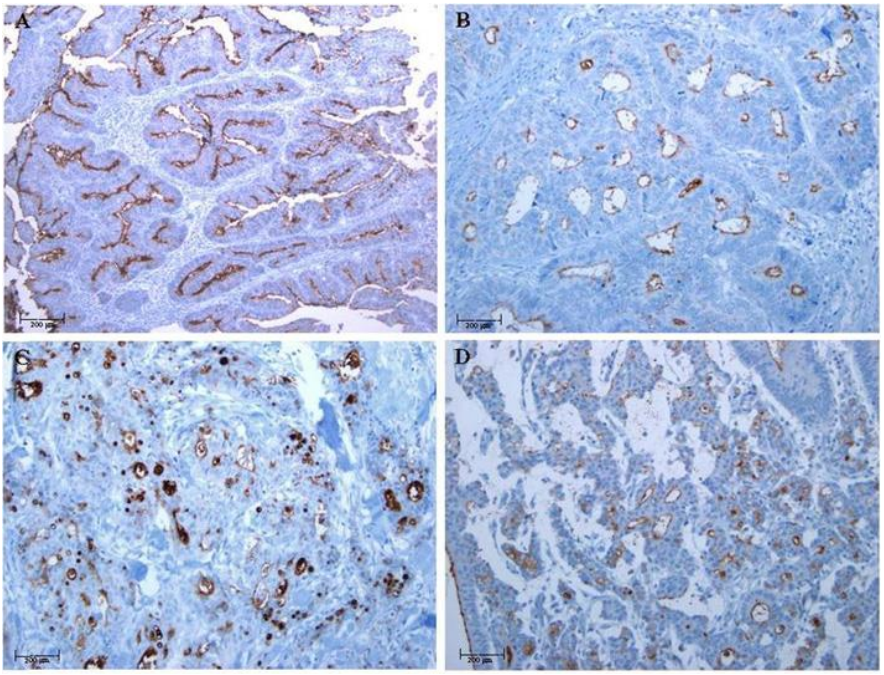

Figure 12. The immunohistochemical expression of CD133 according to histologic differentiation in CRCs. CD133 was expressed along the luminal side of cell membrane in tumors with well differentiation (A), moderate differentiation (B), poor differentiation (C), and (D) mucinous adenocarcinoma.

The expression of CD133 was not homogenous. It was observed in the superficial parts of the tumor as well as mid and deep or invasive parts. There was no difference in CD133 expression at the surface of tumor (Figure 13A) or at the invasive margin (Figure 13B).

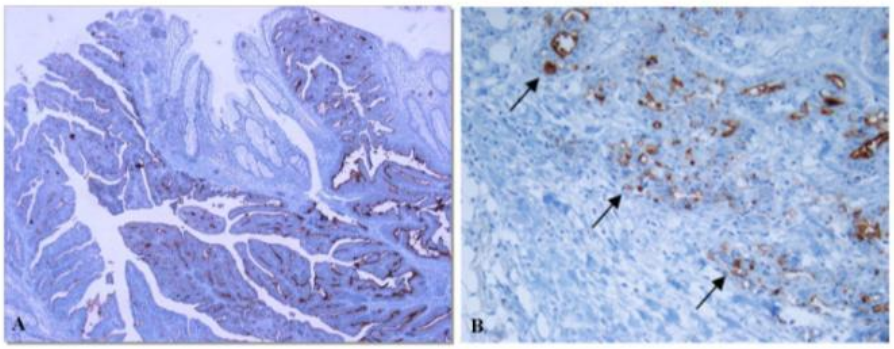

Figure 13. The immunohistochemical expression of CD133 at the surface area and at the invasive margin in CRCs. The expression of CD133 was observed heterogeneously in the tumor area with no specific site-dependence. CD133 expression is seen at the surface of the tumor (A) and at the invasive margin (arrows, B).

(2) Neuroendocrine Neoplasms

CD133 immunohistochemical expression in GEP-NETs was observed as cytoplasmic, luminal or a combination of cytoplasmic & luminal (Table 4). The cytoplasmic staining was either diffuse (Figure 14A & D) or focal (localized only to the apical or basal side of the cytoplasm) (Figure 14B). The luminal staining was observed along the apical side of the cell membrane (apical membrane) (Figure 14C). The combination of cytoplasmic and luminal expression was also observed (Figure 14E).

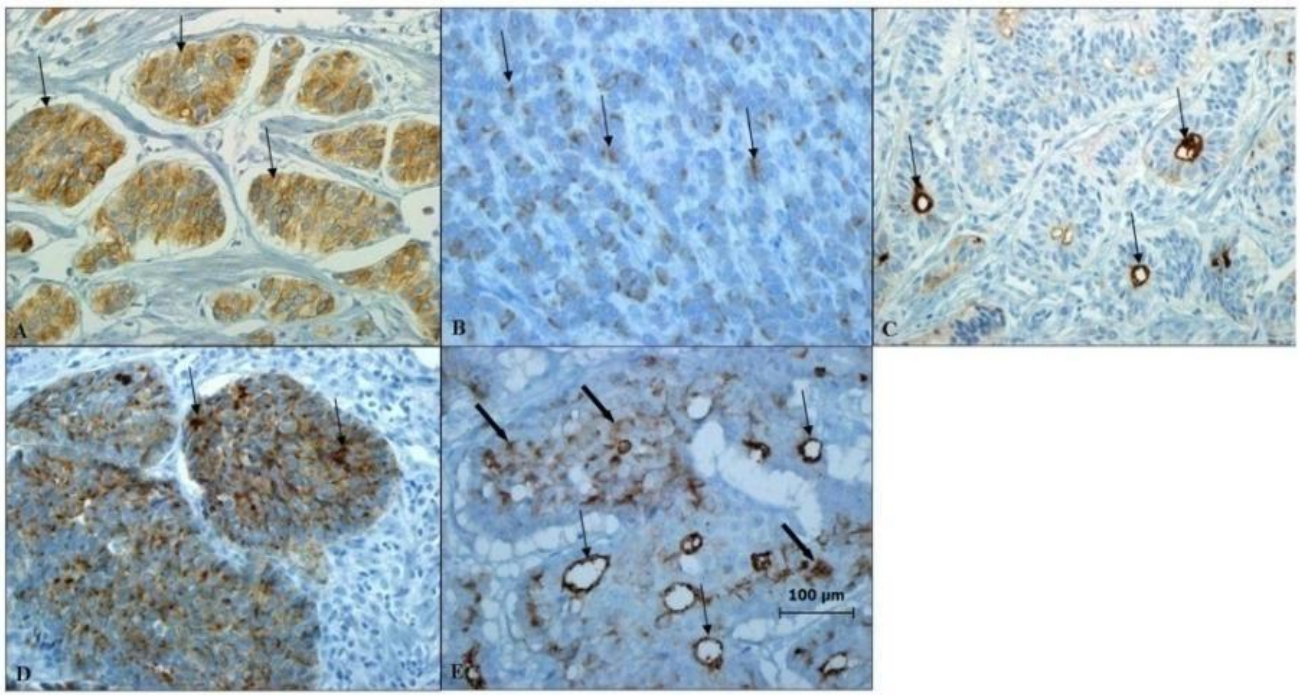

Figure 14. The CD133 immunohistochemical expression patterns according to NEN growth patterns. CD133 was expressed as diffuse cytoplasmic (A & D), focal cytoplasmic (B), luminal (C), and luminal and cytoplasmic (E).

With matched evaluation of CD133 immunohistochemical expression pattern and tumor growth patterns, it was found that tumors with type I (insular or nested) and type IV (poorly differentiated) growth patterns frequently revealed diffuse cytoplasmic expression of CD133, while tumors with type II (trabecular), and type III (glandular or acinar) growth patterns revealed focal & localized cytoplasmic expression and luminal expression of CD133, respectively. MANECs and NETs that revealed insular as well as glandular or acinar growth patterns (type V and type I + III, respectively) showed combination of cytoplasmic and luminal staining for CD133 in each component, respectively. The correlation of the immunohistochemical expression of CD133 according to the growth pattern of tumor cells is summarized in Table 4 and demonstrated in Figure 14.

Table 4. The distribution of the CD133 immunohistochemical expression pattern according to NEN growth

CD133 expression patterns		Tumor growth No. of cases (% of total number of CD133+ cases)				
		I	II	III	IV	V
Cytoplasmic	Diffuse	4 (13.3)	2 (6.6)	1 (3.3)	4 (13.3)	1 (3.3)
	Focal	2 (6.6)	1 (3.3)	-	-	1 (3.3)
Luminal		-	-	1 (3.3)	-	3 (10)
Combined		-	-	1 (3.3)	-	9 (30)
Total		6 (20)	3 (10)	3 (10)	4 (13.3)	14 (46.6)

C. Distribution of CD133 Expression in Tumors of Digestive Tract

(1) Adenocarcinomas

The expression of CD133 was observed in adenocarcinomas of different organs. In gastric adenocarcinomas, 34% (36/107) of patients expressed CD133, while the CD133 was expressed in 47.97% (130/271) of colorectal and 25% (3/12) of pancreatic adenocarcinomas. The expression of CD133 according to tumor differentiation in adenocarcinomas of different organs is shown in Table 5. The expression of CD133 according to different clinicopathologic variables in gastric and colorectal cancers are summarized in Table 6 & 7.

Table 5. CD133 immunohistochemical expression according to differentiation of adenocarcinomas in different organs (%)

	Well	Moderate	Poor	Total
Gastric	7/18 (38.9)	12/34 (35%)	17/55 (30.6)	36/107 (34)
Colorectal	5/16 (31.25)	118/225 (52.44)	7/30 (23.33)	130/271 (47.97)
Pancreatic	0/2 (0)	3/7 (42.85)	0/3 (0)	3/12 (25)

Table 6. The immunohistochemical expression of CD133 in gastric cancer according to clinicopathologic variables

Parameters	All patients (N=107)	CD133 IHC 0%	(1-10%)	(11-50%)	(51-100%)
Sex					
Male	76	26.3	35.5	27.6	10.5
Female	31	45	32.2	19.3	3.2
Differentiation					
Well	18	33.3	27.8	22.2	16.6
Moderate	34	26.4	38.2	26.4	8.8
Poor	55	19	19	14	3
pT-stage					
T1a	28	25	57.1	10.7	7.1
T1b	22	50	22.7	27.3	0
T2	11	27.3	27.3	36.3	9
T3	26	30.7	11.5	38.5	19
T4	20	25	50	20	5
pN-stage					
N0	54	40.7	33.3	18.5	7.4
N1a	13	30.7	15.4	46.1	7.8
N1b	16	18.7	56.3	25	0
N2	18	22.2	33.3	22.2	22.2
N3	6	16.6	33.3	50	0
Lymphovascular invasion					
Yes	2	50	0	50	0
No	55	34.5	36.3	21.8	7.3
Tumor size					
<4cm	57	35.1	42.1	19.3	3.5
>4cm	50	28	26	32	14

Table 7. The immunohistochemical expression of CD133 in CRCs according to clinicopathologic variables

	All patients	CD133 IHC (%)			
	(N=271)	0%	(1-10%)	(11-50%)	(51-100%)
Variables	N	%	%	%	%
Gender					
Male	161	27.95	23.6	22.98	25.46
Female	110	32.72	20	20	27.27
Tumor site					
Colon	150	37.33	22.66	17.33	17.33
Rectum	121	20.66	21.48	27.27	30.57
pTNM stage					
II	122	31.14	25.4	22.13	21.31
III	149	28.85	19.46	21.47	30.2
pT stage					
T1	4	75	0	0	25
T2	6	16.66	66.66	16.66	0
T3	239	29.7	21.75	22.17	26.35
T4	22	27.27	18.18	22.72	31.81
pN stage					
N0	123	30.9	25.2	21.95	21.95
N1	88	29.54	17.04	25	28.4
N2	56	26.78	25	17.85	30.35
N3	4	50	0	0	50
Histologic differentiation					
Well	16	37.5	31.25	18.57	12.5
Moderate	225	25.33	22.22	22.66	29.77
Poor	15	53.33	20	26.66	0
Mucinous	15	66.66	13.33	6.66	13.33
Adjuvant therapy					
Yes	171	28.07	22.22	18.71	30.99
No	100	33	22	27	18

(2) Neuroendocrine Neoplasms

According to Tumor Location:

CD133 was expressed in 20% of gastric NENs, 57.1% of small intestine, 66.6% of appendix, 36.7% of colorectal, 50% of pancreas, 25% of gallbladder and 10% of liver NENs.

According to Histologic Grade:

CD133 was expressed in 31.9% of NET G1, 22.2% of NET G2, 26.1% of NEC (NET G3), and 63.6% of MANECs. The expression of CD133 according to tumor location and histologic grade of GEP-NETs (2010 WHO classification) is summarized in Table 8.

The CD133 expression according to various clinicopathologic parameters is demonstrated in Table 9.

Table 8. CD133 immunohistochemical expression in GEP-NETs according to tumor location and histologic grade

Location	All cases	CD133+ / total				
		NET G1	NET G2	NEC	MANEC	Total
Stomach	15	0/5	0/3	2/5	1/2	3/15 **(20%)**
Duodenum	6	2/3	1/1	1/2	-	4/6 **(66.6%)**
Gallbladder	4	-	-	0/2	1/2	1 /4 **(25%)**
Liver	10	-	-	1/10	-	1/10 **(10%)**
Ileum	1	-	0/1	-	-	0 **(0%)**
Appendix	3	1/1	-	-	1/2	2/3 **(66.6%)**
Colon	8	0/3	-	1/1	3/4	4/8 **(50%)**
Rectum	41	12/35	1/4	1/2	-	14/41 **(34%)**
Total	**90**	**15/47 (31.9%)**	**2/9 (22.2%)**	**6/23 (26.1%)**	**7/11 (63.6%)**	**30/90 (33.3%)**

Table 9. The immunohistochemical expression of CD133 in GEP-NETs according to clinicopathologic variables

Variables	All patients (N=90)	CD133 IHC expression (%)			
		0%	1-10%	11-50%	51-100%
Gender					
Male	55	38.2	20	23.6	18.2
Female	35	54.29	28.6	11.4	5.7
pT stage					
T1	58	37.9	29.3	20.7	12.1
T2	5	20	20	40	20
T3	13	54	23	23	0
T4	3	0	0	0	100
Metastatic	11	91	0	0	9
pN stage					
N0	7	42.8	14.3	14.3	28.6
N1	11	36.4	18.2	27.2	18.2
N2	4	25	25	0	50

Distribution of CD133 IHC Expression According to Neuroendocrine Markers (Synaptophysin and Chromogranin A) Expression

Chromogranin was expressed in 42.5%, 55.5%, 56.5%, and 90.9% of NET G1, NET G2, NEC and MANECs, respectively. On the other hand, synaptophysin was positive in 100%, 90%, 86.9%, and 90.9% of NET G1, G2, NEC and MANECs, respectively. No significant association between CD133 expression and neuroendocrine markers' expression was observed. The distribution of CD133 IHC expression according to the expression of chromogranin and synaptophysin is shown in Table 10.

Table 10. Crosstable of immunohistochemical expression of CD133 in GEP-NETs according to the expression of neuroendocrine markers

Neuroendocrine markers	CD133 IHC expression				Total
	0	1-10%	11-50%	52-100%	(%)
Synaptophysin					
Positive	37	20	17	12	85 (94.4)
Negative	3	1	0	1	5 (5.6)
Chromogranin-A					
Positive	22	6	12	9	48 (53.3)
Negative	18	15	5	4	42 (46.7)

2. Regulatory Mechanism of CD133 Expression in CRCs

A. The mRNA Expression of CD133 in CRCs

The fresh frozen tumor tissues were available in 75 cases (21 colon and 54 rectum) among 271 CRCs. The real-time RT-PCR was performed to semi-quantitatively measure the mRNA expression level of CD133. The mRNA expression of CD133 was found to be significantly directly correlated with the IHC expression level of CD133 (P=0.0257) (Table 11).

B. CD133 Promoter Methylation and CD133 Expression in CRCs

We observed an inverse correlation between CD133 expression and CD133 promoter methylation level. The cases that showed higher CD133 expression by IHC staining were more hypomethylated (Table 11 & Figure 15) while hypermethylation was noted in cases with lower CD133 IHC expression. This inverse correlation between CD133 IHC expression and promoter methylation was statistically significant (P=0.0001). There was an inverse correlation between CD133 mRNA

expression level and CD133 promoter methylation level; however, the correlation between CD133 mRNA expression level and CD133 promoter methylation level was not statistically significant (P=0.1113).

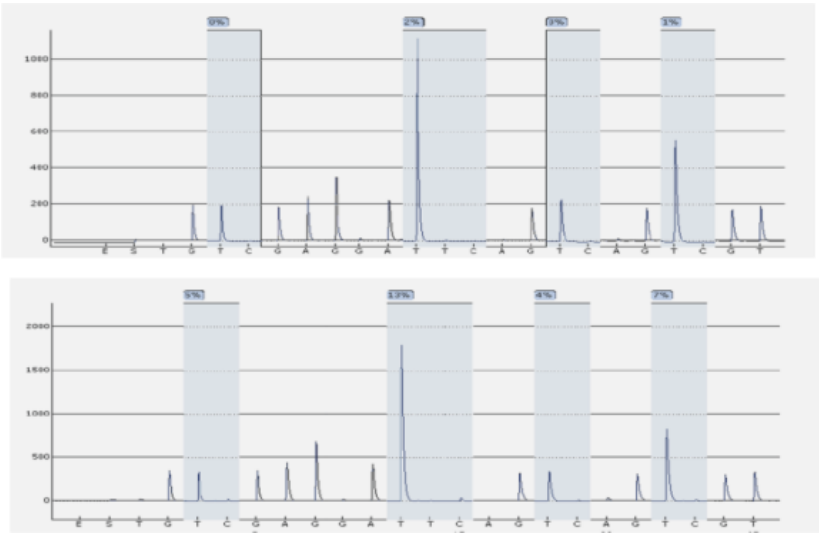

Figure 15. Representative pyrograms from two CRC cases demonstrating methylation status of four CpG sites. The upper case reveals hypomethylation and the lower case shows hypermethylation.

Table 11. Cross-table showing correlation among CD133 IHC staining, mRNA expression and methylation level in CRC

		mRNA	Promoter methylation	IHC
mRNA	Pearson correlation	1	-0.18539	0.25763
	p-value		0.1113	***0.0257****
Promoter methylation	Pearson correlation	-0.18539	1	-0.38263
	p-value	0.1113		***<0.0001****
IHC**	Pearson correlation	0.25763	-0.38263	1
	p-value	0.0257*	<0.0001*	

*p<0.05

IHC: immunohistochemical stain

3. Prognostic Significance of CD133 Expression in Clinical Samples

A. Colorectal Cancer

The clinicopathologic characteristics of CRC patients are summarized in Tables 12-14.

In multivariate analysis, CD133 IHC expression was not an independent prognostic factor in stage II and III colorectal cancer in this study. Overall, CD133 was not correlated to patients' overall survival or disease free survival. However, patients who had received adjuvant therapy had a significantly longer OS time compared to those who did not receive adjuvant therapy ($p<0.0001$). And among the group with CD133+ tumors in this study (n=130), patients with adjuvant therapy (n=85) had a better OS compared to those without adjuvant therapy (n=45) ($p<0.0001$, HR 0.125, 95% CI 0.052-0.299, Figure 16). However, the CD133- tumors (≤10%, n=141) did not show significant difference in OS between the two groups (adjuvant therapy, n=86 vs. no adjuvant therapy n=55, P=0.055, HR 0.500, 95% CI

0.247-1.015, Figure 17). However, there was no significant correlation between CD133 IHC expression and DFS according to adjuvant therapy (P=0.2451).

Table 12. The clinicopathologic characteristics of CRC patients

Parameter		Number of patients
Gender		
	Male	161
	Female	110
TNM Stage		
	II	122
	III	149
pT		
	1	4
	2	6
	3	239
	4	22
pN		
	N0	123
	N1	88
	N2	56
	N3	4

Table 13. The CRC patients profile according to adjuvant therapy status

Location	Adjuvant therapy		No adjuvant therapy		Total
	Stage II	Stage III	Stage II	Stage III	Stage II+III
Colon	40	51	38	21	150
Rectum	23	57	21	20	121
Total	63	108	59	41	271

Table 14. The CRC patients profile according to tumor differentiation

Location	Well (%)	Moderate (%)	Poor (%)	Total (%)
Colon	9 (6.00)	121 (80.66)	20 (13.33)	150 (100)
Rectum	7 (5.78)	104 (85.95)	10 (8.26)	121 (100)
Total	16 (5.90)	225 (83.02)	30 (11.07)	271 (100)

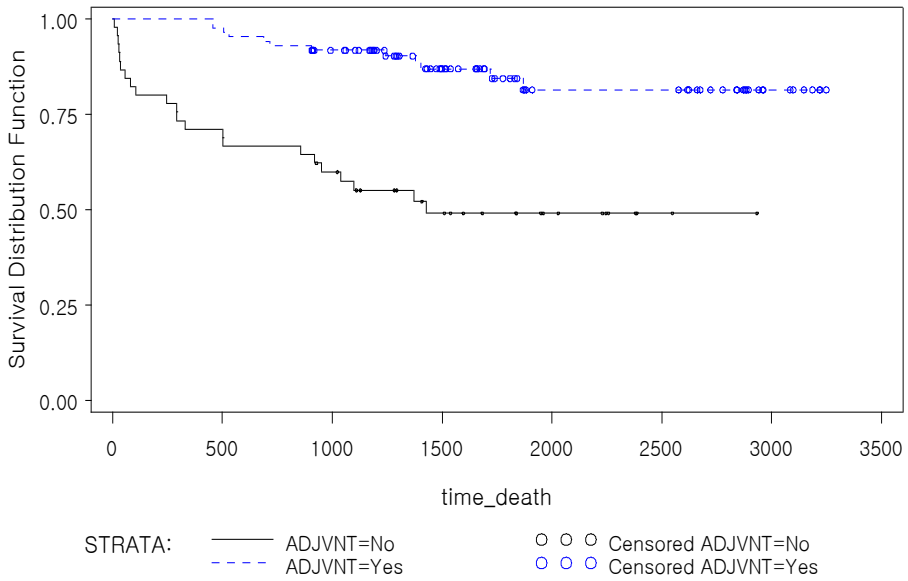

Figure 16. Kaplan-Meier survival curves in the CD133+ (≥10% expression) colorectal cancer patients according to adjuvant therapy status. The patients with CD133+ CRC had a significantly shorter OS if they had not received adjuvant therapy (P=0.0001).

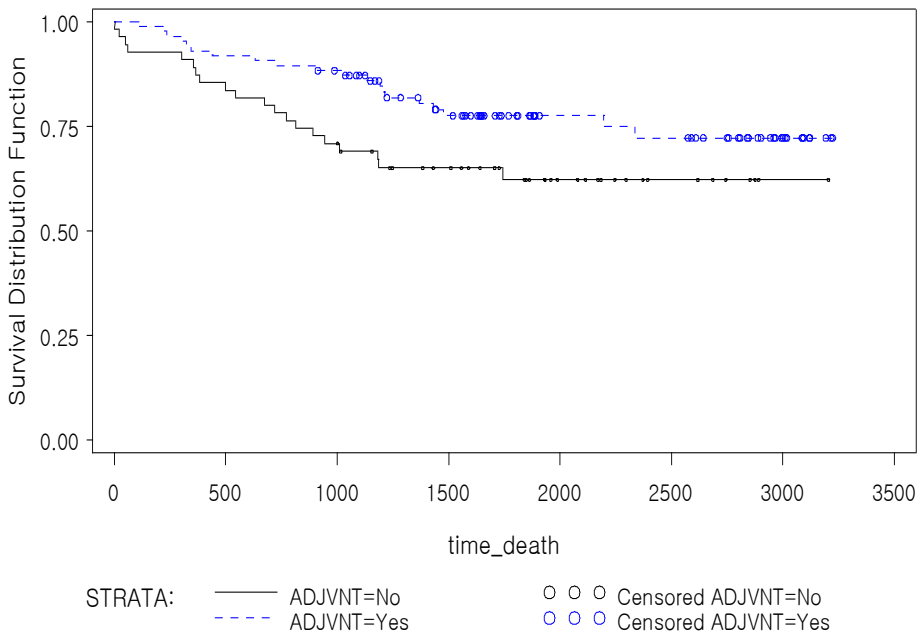

Figure 17. Kaplan-Meier survival curves in the CD133- (<10% expression) colorectal cancer patients according to adjuvant therapy status. There was no statistically significant difference in OS according to the adjuvant therapy status in CD133- tumors (P=0.055).

In χ^2- analysis and Mantel-Haenszel test, CD133 IHC expression was significantly different according to tumor location (colon vs. rectum) (P=0.0158, Figure 18) and histologic differentiation (P=0.0378, Figure 19). The moderately differentiated tumors and rectal tumors showed more CD133 expression than others. There was no significant relationship between CD133 IHC expression and other clinicopathologic variables studied such as sex (P=0.8233, Figure 20), pTNM stage (P=0.3598, Figure 21), invasion depth (P=0.160, Figure 22), and lymph node metastasis (P=0.346, Figure 23).

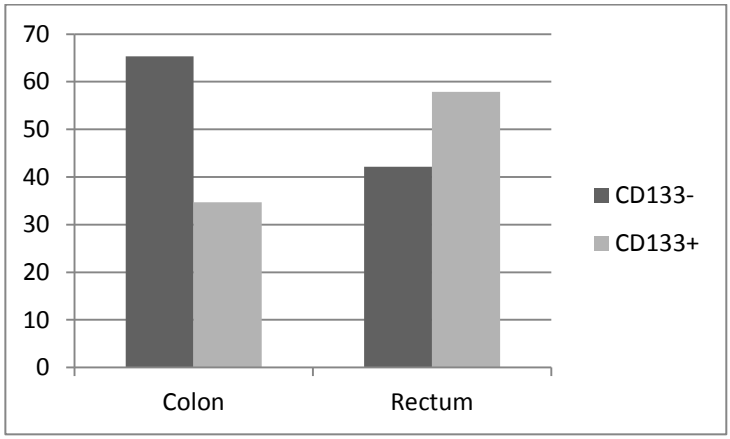

Figure 18. CD133 immunohistochemical expression in CRCs according to tumor location. CD133 was expressed more in rectal tumors compared to colon (P= 0.0158).

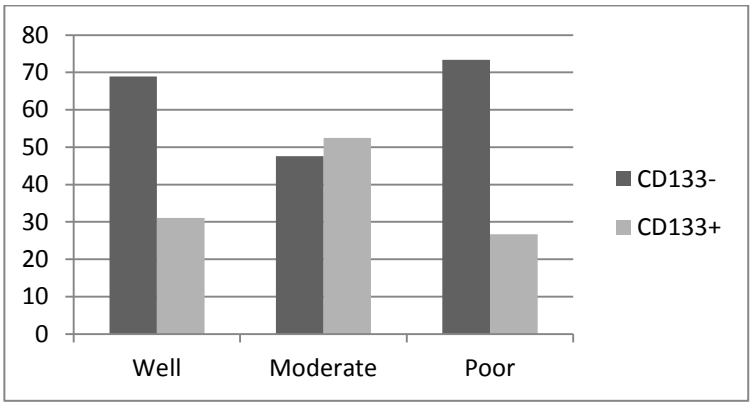

Figure 19. CD133 immunohistochemical expression in CRCs according to the histologic differentiation. Tumors with moderate differentiation showed more positivity for CD133 compared to well and poorly differentiated ones (P= 0.0378).

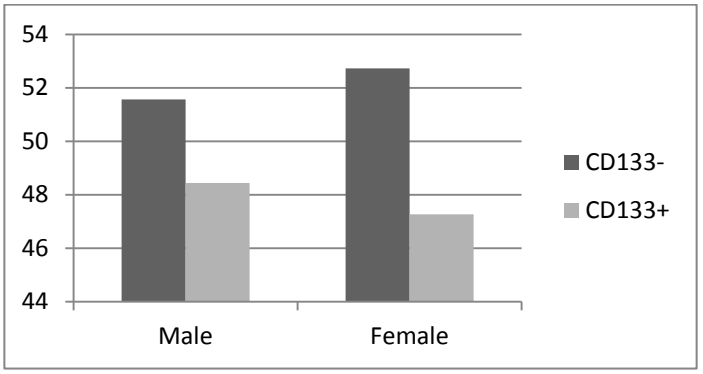

Figure 20. The distribution of CD133 immunohistochemical expression according to gender in CRCs. No difference in CD133 expression according to gender was observed (P= 0.8233).

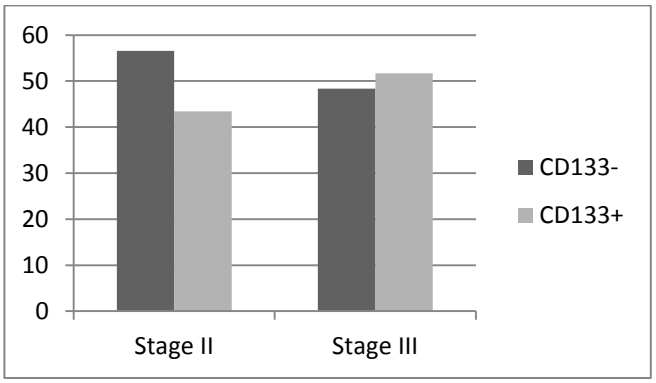

Figure 21. CD133 immunohistochemical expression according to tumor stage in CRCs. There was no significant difference of CD133 expression between stage II and III. (P= 0.3598).

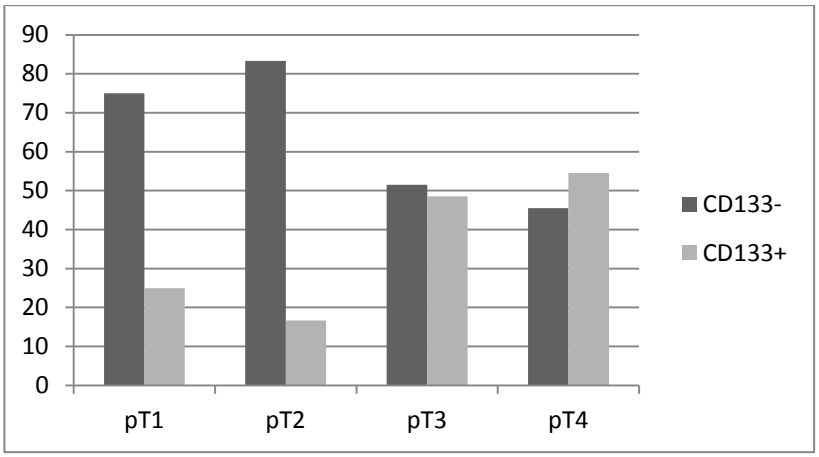

Figure 22. CD133 immunohistochemical expression according to the invasion depth in CRCs. There was no significant association between CD133 expression and depth of invasion (P= 0.160).

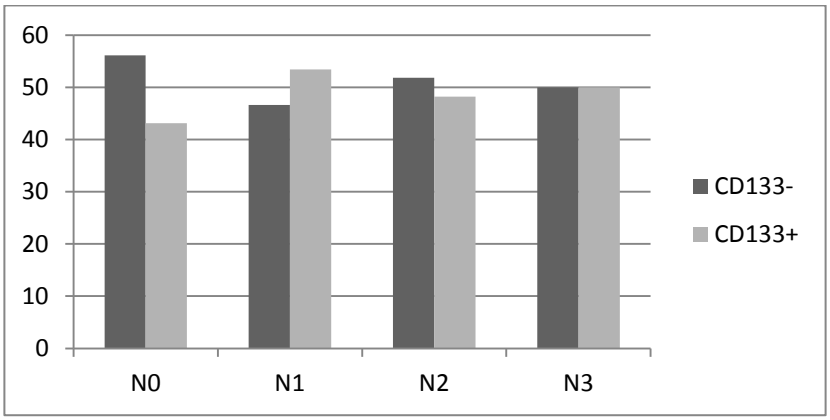

Figure 23. CD133 immunohistochemical expression according to lymph node metastasis in CRCs. No association between CD133 expression and lymph node metastasis was observed (P= 0.346).

B. Neuroendocrine neoplasms

The survival analysis, adjusted for age and tumor stage, was performed for 23 patients with NECs. Six out of 23 NECs were positive for CD133. No prognostic significance of CD133 immunohistochemical expression was found in these patients (P=0.97, Figure 24).

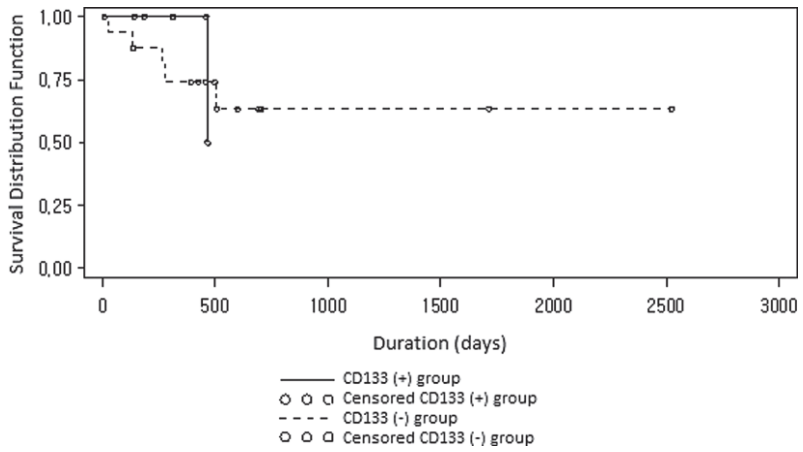

Figure 24. CD133 immunohistochemical expression and overall survival in patients with NEC. There is no significant correlation between the CD133 expression and overall survival in NEC patients. (P=0.97).

4. Effects of CD133 Inhibition in CRC Cell Lines by siRNA Transfection

A. Expression of CD133 mRNA in CRC Cell Lines

The expression of CD133 mRNA was initially evaluated in four colorectal cancer cell lines (Caco-2, HT-29, HCT-15 and DLD-1) by RT-PCR. Similar to previous reports, CD133 mRNA expression was observed in Caco-2, HT29 and DLD-1 cell lines. However, HCT-15 cells showed weak CD133 mRNA expression (Figure 25). Therefore for CD133 siRNA transfection, only Caco-2, HT-29, and DLD-1 cell lines were selected.

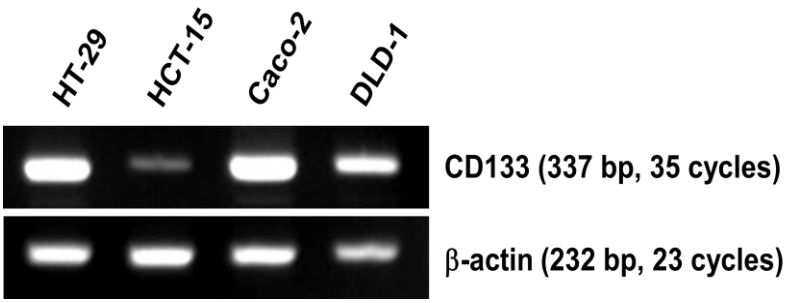

Figure 25. CD133 mRNA expression in CRC cell lines. Strong CD133 mRNA expression was observed in HT-29, Caco-2, and DLD-1 cells while HCT-15 cells showed weak CD133 mRNA expression.

B. Inhibition of CD133 mRNA Expression by siRNA Transfection in CRC Cell Lines

The siRNA transfection was performed to Caco-2, HT-29 and DLD-1 cell lines. We could successfully achieve CD133 gene knock-down in DLD-1 cells with 10 nM and 20 nM concentration and in Caco-2 cells with 10 nM, 20 nM, and 30 nM of siRNA mixture. Due to technical problems, CD133 siRNA transfection was not achieved in HT-29 cell line (bad condition of the purchased cells).

Forty eight hours after siRNA transfection, the expression of CD133 mRNA was evaluated in DLD-1 and Caco-2 cells by RT-PCR in four groups with different CD133 sequence targets (Figure 26). The expression level of CD133 mRNA was further semi-quantitatively evaluated by qRT-PCR in DLD-1 cell line in all four groups at 48 hour (Figure 27), and in siRNA1 transfection group ($CD133^{siRNA1}$) at 48 hour and 72 hour post-transfection (Figure 28) and in Caco-2 cells in all four groups with different siRNA concentration at 48 hour and 72 hour post-transfection (Figure 29). For further analysis, only the first siRNA transfection group ($CD133^{siRNA1}$) was used.

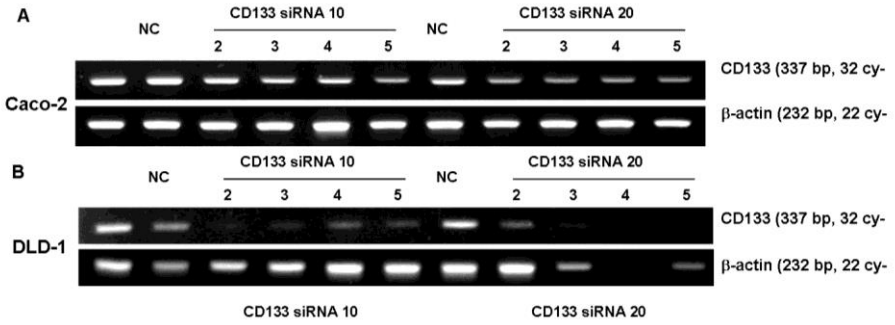

Figure 26. CD133 mRNA expression by RT-PCR in two CRC cell lines after siRNA transfection. The CD133 mRNA was successfully blocked by siRNA transfection in (A) Caco-2 cell line and (B) DLD-1 cell line with both 10 nM and 20 nM siRNA concentrations at 48 hour post-transfection. NC represents normal control group, while 2, 3, 4, and 5 represents group 1, 2, 3, and 4 (different sequences against which siRNA was transfected).

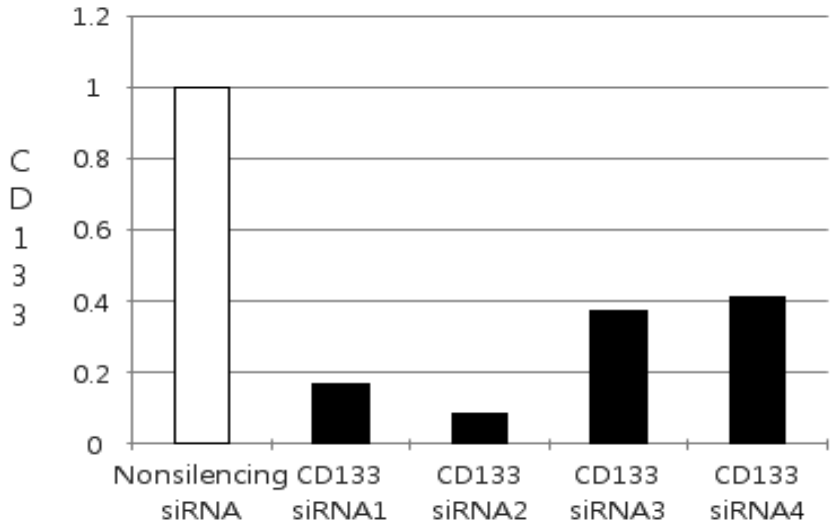

Figure 27. CD133 mRNA expression in DLD-1 cells at 48 hour after siRNA transfection by qRT-PCR. The expression of CD133 mRNA is greatly decreased in all four groups (these groups represent different parts of the CD133 gene sequence against which siRNA was transfected) by siRNA transfection at 10 nM concentration. In $CD133^{siRNA1}$ and $CD133^{siRNA2}$ groups, the CD133 mRNA was knocked-down to 85-90% while in $CD133^{siRNA3}$ and $CD133^{siRNA4}$ group, the CD133 mRNA level was knocked-down up to 60%.

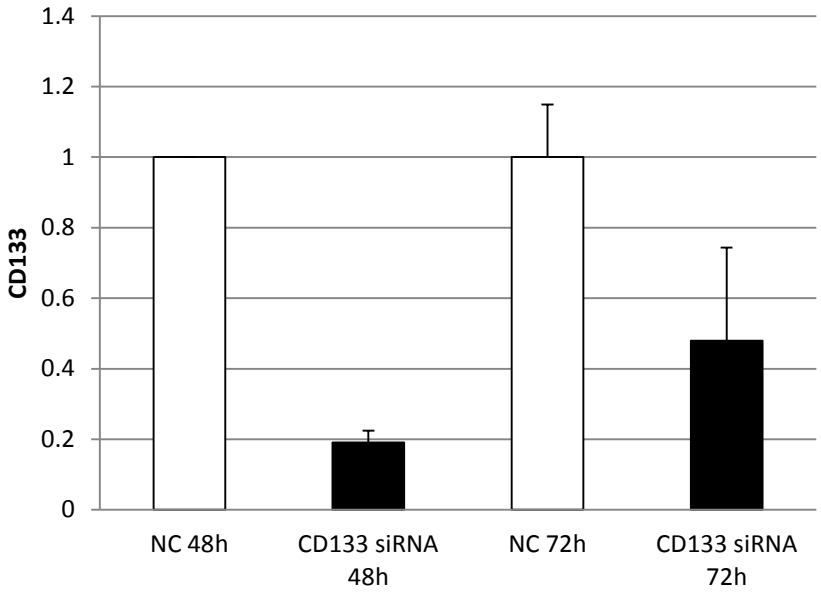

Figure 28. CD133 mRNA expression in DLD-1 cells at 48 hour and 72 hour after siRNA1 transfection by qRT-PCR. The expression level of CD133 mRNA is greatly decreased both at 48 and 72 hour after CD133 siRNA1 transfection compared to control (NC) groups.

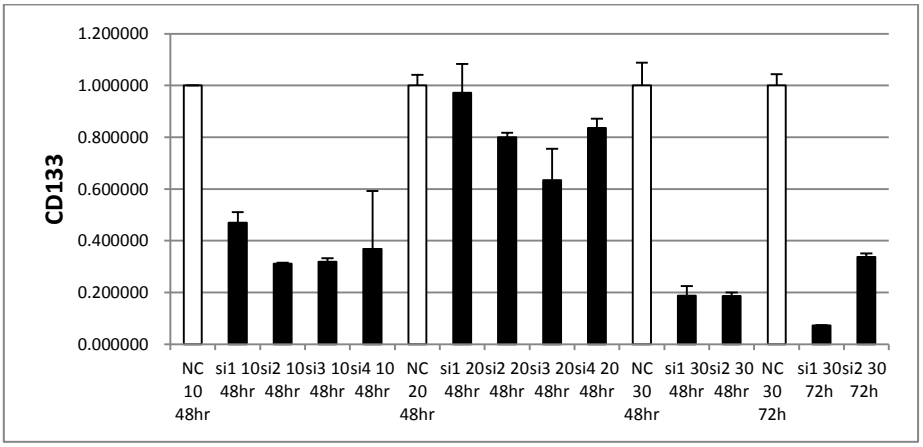

Figure 29. CD133 mRNA expression in Caco-2 cells by qRT-PCR at 48 hour and 72 hour after siRNA transfection. The expression of CD133 mRNA in Caco-2 cells is greatly decreased in all four groups (these groups represent different parts of the CD133 gene sequence against which siRNA was transfected) by siRNA transfection at 10 nM and 20 nM concentration and in two groups with 30 nM concentration at 48 hour and 72 hour post-transfection.

C. Evaluation of Genes Related with Chemoresistance (*ABCG2* and *AKT1*) in CRC Cell Lines after CD133 Inhibition by siRNA Transfection

(1) *ABCG2* mRNA Expression after CD133 siRNA Transfection in DLD-1 and Caco-2 Cell Lines

The mRNA expression level of *ABCG2* was evaluated before and 48 and 72 hour after CD133 knock-down by siRNA in DLD-1 and Caco-2 cell lines. As observed in Figure 30, the mRNA expression of *ABCG2* was gradually increased after CD133 knock-down at 48 hour and 72 hour post-transfection compared to control (NC) groups in DLD-1 cells. Similarly, the mRNA expression of *ABCG2* greatly increased after CD133 siRNA transfection in Caco-2 cells at different concentrations of siRNA at 48 hour but was reduced at 72 hour post-transfection (Figure 31).

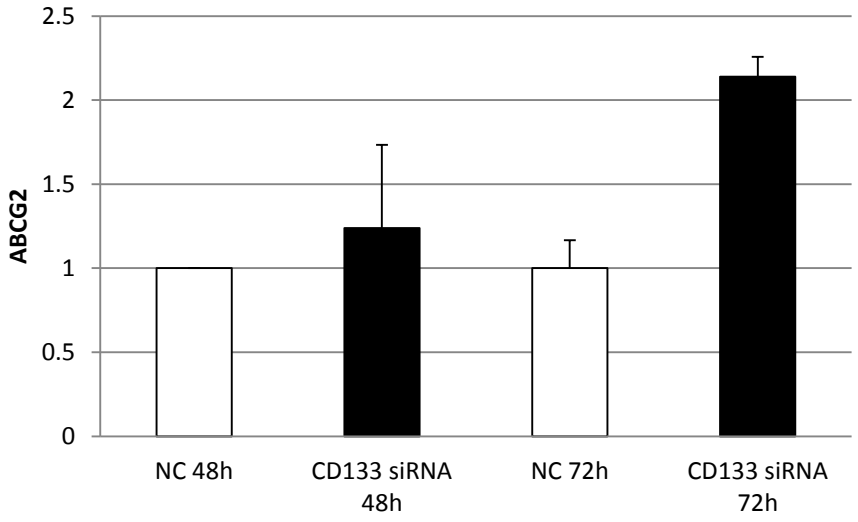

Figure 30. Expression of *ABCG2* mRNA in DLD-1 cells by qRT-PCR at 48 hour and 72 hour after CD133 siRNA1 transfection. The mRNA expression level of *ABCG2* was gradually increased after CD133 knock-down at 48 hour and 72 hour after CD133 siRNA transfection in DLD-1 cells compared to control (NC) groups.

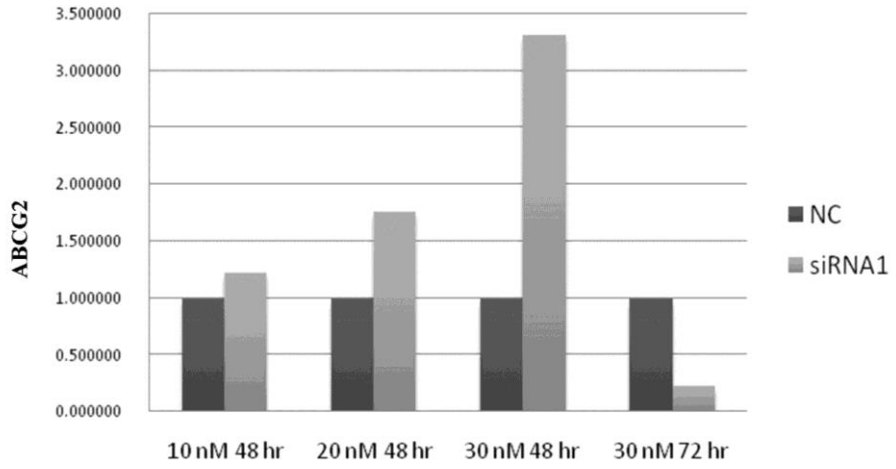

Figure 31. Expression of *ABCG2* mRNA in Caco-2 cells by qRT-PCR at 48 hour and 72 hour after CD133 siRNA1 transfection. The mRNA expression level of *ABCG2* was increased after CD133 knock-down at 48 hour with different concentrations of siRNA and decreased at 72 hour after CD133 siRNA transfection in Caco-2 cells compared to control (NC) groups.

(2) *AKT1* mRNA Expression after CD133 siRNA1 Transfection in DLD-1 and Caco-2 Cell Lines

For this purpose, the mRNA expression level of *AKT1* was quantitatively evaluated by qRT-PCR in DLD-1 and Caco-2 cell lines before and 48 hour and 72 hour after CD133 knock-down by siRNA transfection. The mRNA expression of *AKT1* was not changed at 48 hour but increased at 72 hour after CD133 knock-down compared to control (NC) groups in DLD-1 cell lines (Figure 32). Similarly, in Caco-2 cell line, the mRNA expression level of *AKT1* increased at 48 hour post-transfection along with the increase in the siRNA concentration but decreased at 72 hour post-transfection compared to control groups (Figure 33).

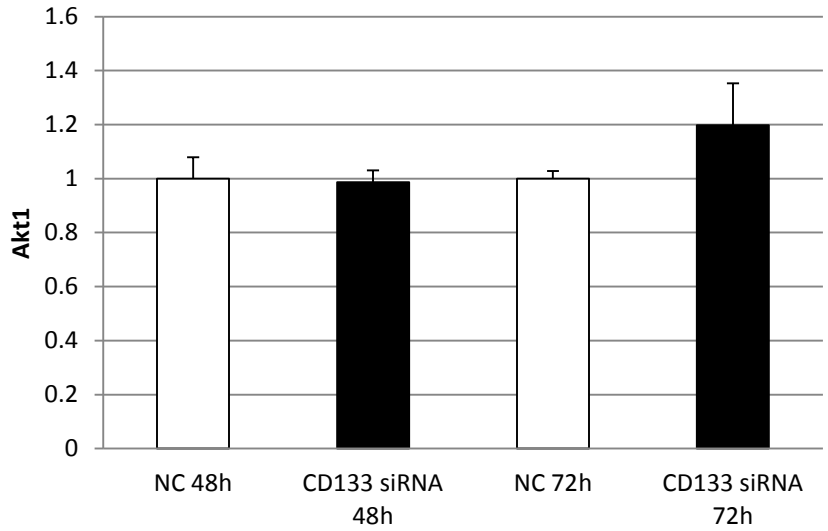

Figure 32. Expression of *AKT1* mRNA in DLD-1 cells by qRT-PCR at 48 and 72 hour after CD133 siRNA1 transfection. The expression level of *AKT1* mRNA1 was not changed at 48 hour but was increased at 72 hour post-transfection in DLD-1 cells compared to control (NC) groups.

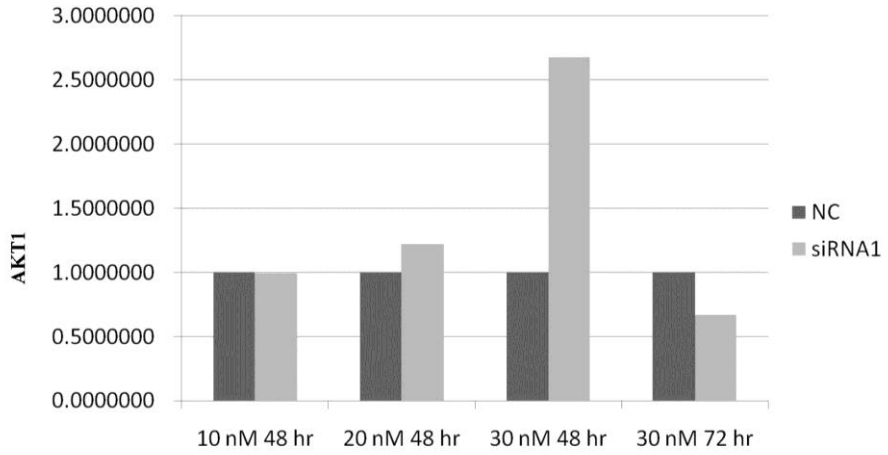

Figure 33. Expression of *AKT1* mRNA in Caco-2 cells by qRT-PCR at 48 and 72 hour after CD133 siRNA1 transfection. The mRNA expression level of *AKT1* was gradually increased along with the increase in siRNA concentration at 48 hour but reduced at 72 hour post-transfection in caco-2 cells compared to control (NC) groups.

D. Evaluation of Genes Related with Cell Proliferation and Apoptosis (*β-catenin* and *Survivin*) in CRC Cell Lines after CD133 Inhibition by siRNA1 Transfection

(1) *β-catenin* mRNA Expression after CD133 siRNA1 Transfection in DLD-1 and Caco-2 Cell Lines

In DLD-1 cell line, the mRNA expression level of *β-catenin* was quantitatively evaluated by qRT-PCR before and 48 hour and 72 hour after CD133 knock-down by siRNA. As observed in Figure 34, the expression of *β-catenin* mRNA was decreased after CD133 knock-down both at 48 hour and 72 hour post-transfection compared to control (NC) groups. Similarly, the mRNA expression of *β-catenin* was decreased both at 48 and 72 hour post-transfection in Caco-2 cells compared to control groups (Figure 35).

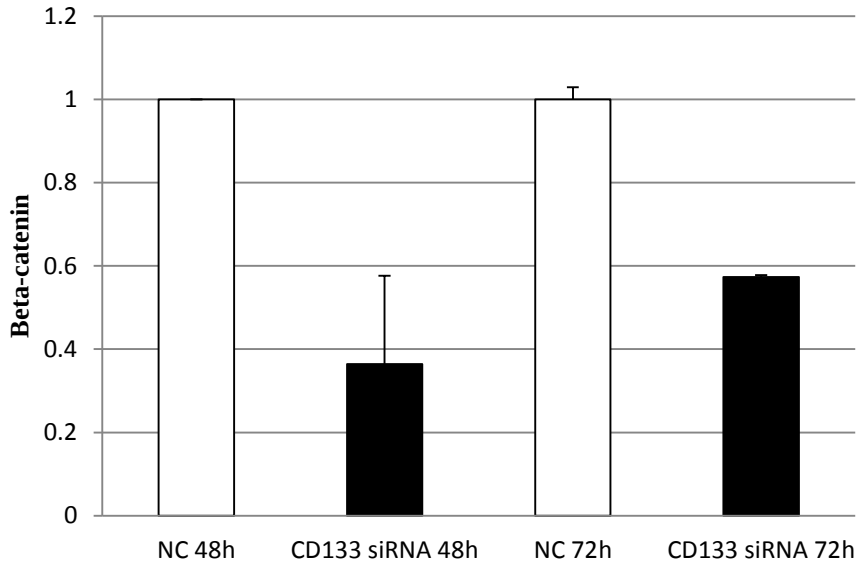

Figure 34. Expression of *β-catenin* mRNA in DLD-1 cells by qRT-PCR at 48 and 72 hour after CD133 siRNA1 transfection. The expression of *β-catenin* mRNA was decreased after CD133 knock-down by siRNA1 in DLD-1 cells compared to control (NC) groups.

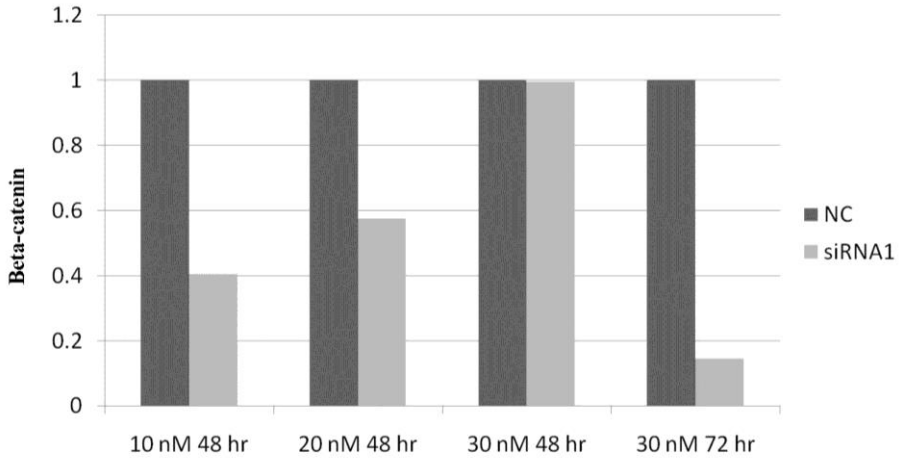

Figure 35. Expression of *β-catenin* mRNA in Caco-2 cells by qRT-PCR at 48 and 72 hour after CD133 siRNA1 transfection. The expression of *β-catenin* mRNA was decreased after CD133 knock-down by siRNA at 48 hour as well as 72 hour post-transfection in Caco-2 cells compared to control (NC) groups.

(2) *Survivin* mRNA Expression after CD133 siRNA Transfection in DLD-1 and Caco-2 Cell Lines

The mRNA expression level of *Survivin* was quantitatively evaluated by qRT-PCR before and 48 and 72 hour after CD133 knock-down by siRNA in DLD-1 and Caco-2 cell lines. As observed in Figure 36, the expression of *Survivin* mRNA was decreased after CD133 knock-down both at 48 hour and 72 hour post-transfection compared to control (NC) groups. In Caco-2 cells, however the expression of *Survivin* mRNA was increased at different concentrations at 48 hour while it was decreased at 72 hour post-transfection (Figure 37).

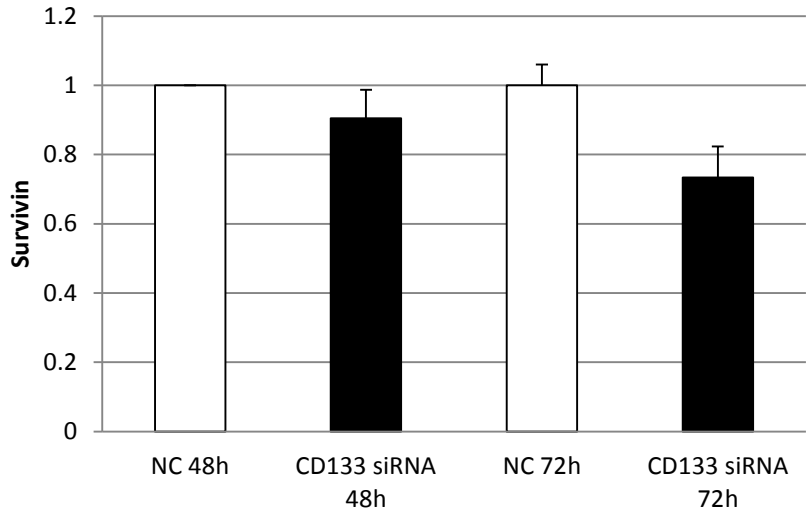

Figure 36. Expression of *Survivin* mRNA in DLD-1 cells by qRT-PCR at 48 hour and 72 hour after CD133 siRNA1 transfection. The mRNA expression level of *Survivin* was gradually decreased after CD133 knock-down by siRNA in DLD-1 cells both at 48 hour and 72 hour after transfection compared to control (NC) groups.

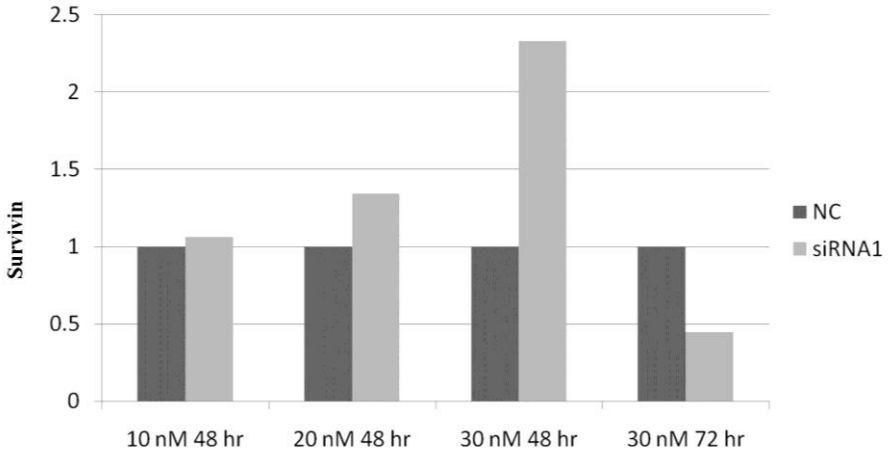

Figure 37. Expression of *Survivin* mRNA in Caco-2 cells by qRT-PCR at 48 hour and 72 hour after CD133 siRNA1 transfection. The mRNA expression level of *Survivin* was gradually increased after CD133 knock-down by siRNA in Caco-2 cells at 48 hour post-transfection at different siRNA concentrations but decreased at 72 hour after transfection compared to control (NC) groups.

IV. DISCUSSION

In the human embryo, CD133 is expressed in various developing epithelia.[34] CD133 expression seems to be down-regulated in most adult epithelial tissues with few exceptions such as pancreatic ducts that remain to express CD133 in adulthood.[60] In adult organ systems, CD133 remains detectable only in very rare cells with stem cell properties.

The expression of CD133 as a CSC marker in different solid tumors has been previously reported, although they have been contradictory and controversial. Overall, it is believed that CSCs exist in the CD133 (+) subpopulation of tumors. And CD133- positivity has gained much importance for predicting worse prognosis in several malignancies.

The CSC theory finds a concrete basis of rationality in colorectal cancer owing to the fact that colon epithelium physiologically regenerates and is shed periodically over a short span of time not compatible with traditional model of carcinogenesis according to which cells ought to be more long-lived and suffer several mutations

and genetic alterations to be able to convert to a cancer cell.[61-62]

We described the distribution and prognostic significance of CSC marker, CD133 expression in adenocarcinomas and NENs of various organs of digestive tract. CD133 was expressed in 47.97% of CRCs, 34% of gastric and 25% of pancreatic cancers. It is higher than previously reported. Previous studies have revealed controversial findings regarding the pattern (cytoplasmic vs. membranous) and distribution of CD133 IHC staining. These differences in previous reports could arise from using different antibodies, tissue samples (cell lines vs. human tissue), methods of detection (IHC expression, flow cytometry or PCR based techniques),[63] and tissue sampling method (tissue microarray vs. individually mounted tissue slides) and method for scoring the positivity of CD133 IHC expression.

To avoid the scoring bias and in view of a recent paper in which CD133 positivity was quantitatively graded[64], we used four-tiered scoring method comprising 0 (totally negative cases), 1+, 2+, and 3+ cases (CD133+ cells covering 1-10%, 11-50%, and 51-100%, of the tumor area, respectively). Nevertheless, we considered the 2+ and 3+

groups as CD133 positive.

On the other hand, due to the fact that CD133 is not homogenously expressed and inability of microarray to fully represent the whole tumor, we used individually-mounted whole-block tissue slides for IHC analysis. Furthermore, to decrease the staining bias, we used automated machine for all procedure of IHC staining.

Additionally, we herewith for the first time described the pattern and distribution of CD133 in GEP-NETs as well as the non-neoplastic epithelial cells of gastrointestinal tract, pancreas and liver.

CD133 associates with cholesterol-rich membrane microdomains "lipid rafts" and localizes to special subdomains of the plasma membrane of cells.[65] Recent studies have shown the two distinct staining patterns for CD133: apical/endoluminal membranous staining and cytoplasmic staining. It was suggested that luminal expression of CD133 is observed in the malignant glandular epithelia while cytoplasmic positivity is seen in the non-epithelial tumors.[66] Contrary to that, reports from different studies including our present work[67-68] have verified the cytoplasmic expression of CD133 in epithelial

malignancies.

The significance of pattern of expression has been controversial. Some studies suggested that cells with cytoplasmic CD133 staining are putative CSCs.[69-70] However, majority of studies that claim the CSC marker role for CD133 have reported membranous staining pattern.[65, 71] Localization of this molecule in different cellular compartments by immunohistochemistry has led to the assumption that it may have specific functional role in the membrane and cytoplasm. The cytoplasmic expression is thought to happen when the molecule is transported from the plasma membrane into the cytoplasm during endocytosis.[70] Membranous localization has been previously described as being important for cell movement, which is associated with important processes such as chemotaxis, embryonic development, mobility and the under-controversy stem cell property.[72] Similarly, it was suggested that localized CD133 distribution in the cell membrane is a mechanism of asymmetric CD133 segregation during mitosis in glioblastoma.[73] Molecular genetic analysis of autosomal recessive retinal degeneration has demonstrated that the affected individuals had a frame-shift mutation in CD133 with premature termination of

translation, and the truncated protein could not be transported to the cell surface. Absence of membranous CD133 expression in a previous study on hepatocellular carcinoma was concluded as the accumulation of such truncated CD133 protein that might result in rapid degradation. Similar phenomenon has been already observed for the epidermal growth factor receptor in cultured cells.[74] Another possible mechanism discussed for the justification of above-mentioned claim has been pointed out as the failure of transportation and insertion of newly synthesized molecules within the Golgi apparatus into the membranes.[75] Which pattern of expression represents the thorough nature of CD133 and its functional relevance is yet to be rectified.

In line with findings of previous studies on colon[71, 76-77], tumors with moderate differentiation showed higher level of CD133 IHC expression compared to poorly differentiated tumors and mucinous adenocarcinomas. No difference was noted in IHC expression between superficial and deep areas (Figure 13). CD133 expression was similarly observed in adenocarcinomas of stomach and pancreas. We rarely found unequivocal luminal staining at the crypt base in non-neoplastic colonic mucosa around the tumor, similar to the results of

previous studies.[26, 38, 78] In comparison with the CD133 IHC expression of non-neoplastic colorectal mucosa, there are relatively frequent CD133 expression in the luminal border of non-neoplastic mucosa of stomach (pyloric type glands only), duodenum (Brunner's glands), liver (bile ducts only), and pancreas (small pancreatic ducts and centroacinar cells). The expression of CD133 in non-neoplastic mucosa of jejunum and ileum is rare and is similar to non-neoplastic colorectal mucosa (rare cells at crypt base). Given these results, further study seems to be required to clarify whether CD133 is an adequate cancer stem cell marker or not.

Importantly, we did not observe CD133 positivity in the non-neoplastic neuroendocrine cells, neither in those that are scattered throughout the gastrointestinal tract mucosa nor in the pancreatic islets. The NENs in different organs arise from the neuroendocrine cells that are located in that area and they reveal the same immunohistochemical profile as the cells that they arise from. Lack of CD133 positivity indicates that CD133 is not a marker for normal neuroendocrine cells. On the contrary, the expression of CD133 was observed in NENs of various organs and of different histologic grade. The IHC expression

of CD133 in GEP-NETs is hence not a mere reflection of the cytoplasmic content of endocrine granules and histologic differentiation. This is the first report to show the expression of CD133 in the GEP-NETs and the first report to show that not only poorly differentiated NECs (small cell carcinomas) but also the well differentiated NETs (NET G1 and NET G2) express CD133. The significance of such expression however remains to be clarified in future in-depth and comprehensive studies.

In this study, we used monoclonal antibody against the CD133/1 or AC133, one of the two epitopes of the CD133 protein. The other epitope is AC141. Although, the monoclonal antibodies against these two epitopes have been interchangeably used to purify and characterize various stem and progenitor cells[32], there is rarely discordant expression of the AC133 and AC141 epitopes observed such as in a study on patients with myelodysplastic syndrome and acute myelogenous leukemia.[73] Initially generated to target CD133 surface antigen, AC133 has been lately used to identify cancer stem cells in several types of solid tumors. It was demonstrated that the AC133 epitope rather than the CD133 protein seems to be restricted to the

stem cells.[27] In a recent paper, using the two widely used monoclonal antibodies (AC133 and Ab19898), it was found that the AC133 epitope expression acted as an adverse prognostic factor in colorectal cancer patients, matching with the CSC characteristics.[79]

In addition, few important factors need to be considered while using monoclonal antibodies against an epitope of CD133. First of all, there is little known about the characteristics of the two epitopes detected by the monoclonal antibodies. Secondly, these epitopes are suggested to be glycosylated (however the supporting evidence for this claim is not well verified in the existing literature)[80] and this glycosylation is reported to be down-regulated upon differentiation of epithelial cells. An additional confusing factor is the presence of alternatively spliced variants of *CD133*. There in human CD133 gene exist at least 37 exons and several alternatively spliced forms.[28] Although, there is little knowledge about the existence of alternatively spliced CD133 isoforms that lack the AC133 or AC141 epitopes, the epitope-negative cells (cells that are negative against the monoclonal antibodies such as the CD133 negative cases in our study) may not solely and necessarily mean CD133 negativity in the absence of proper verification of CD133

protein or mRNA levels.[80] Additionally, it was recently concluded that AC133 does not recognize a glycosylated epitope, in contrast to previous suggestions[27, 81] and described that differential splicing is also not the cause of differential AC133 recognition. However, it remains for the future studies to comparatively use antibodies against all known glycosylated and non-glycosylated epitopes of CD133 to draw a confident conclusion over the validity of the tested monoclonal antibodies.

To validate our IHC results in CRCs, we also evaluated CD133 mRNA expression in 75 cases out of 271 cases which had available fresh frozen tissue. There was a significant direct correlation between mRNA expression and CD133 IHC expression (P=0.0257). Since the CD133 IHC expression happened to be observed only in tumors of digestive tract (with the exception of rare cells in non-neoplastic mucosa) and there is significant direct correlation between the IHC and mRNA expression level, there may be minute chance of missing isoforms of CD133 (if any) that may lack epitope-immunoreactivity via our IHC staining. Unfortunately, we could not evaluate the prognostic significance of CD133 mRNA expression according to the

adjuvant therapy status due to limitation in number of cases with available fresh frozen tissue.

To verify the regulatory mechanism of CD133 expression, we performed methylation analysis and found significant inverse correlation between CD133 expression and promoter methylation level ($p<0.0001$). This finding is concordant with previous study on colon cancer cell lines.[1] But, the correlation of CD133 mRNA with methylation was not statistically significant. The lack of statistical significance in correlation between the level of CD133 mRNA and promoter methylation suggests that other factors may be additionally involved in the regulation of CD133 expression.

We studied the correlation between CD133 IHC expression and patients' survival in stage II and III CRCs as well as NECs.

In colorectal cancer patients, although CD133 IHC expression was not correlated with OS (P=0.9778) and DFS (P=0.2451), the group of patients with CD133+ tumors showed better OS if they received adjuvant therapy compared to patients without adjuvant therapy in the Log-Rank test. Multivariate analysis adjusted with age and stage also

showed statistical significance between two groups ($p<0.0001$, HR 0.125, 95%CI 0.052-0.299). However the patients with CD133- tumors did not show any difference in OS between two groups (P=0.055, HR 0.500, 95% CI 0.247-1.015). Therefore the adjuvant therapy can be of benefit for patients with CD133+ tumor in contrast to patients with CD133- one. This stands against the notion that tumors with high CD133 positivity are resistant to adjuvant therapy.[78] Our results are in support of a recent paper which has demonstrated that CD133+ tumor cells are not more resistant to chemotherapy than CD133- tumor cells.[82] Noteworthily, this finding asks for further elucidation of the matter and moreover notifies that stage II and III colon cancer patients with CD133 IHC expression may benefit from adjuvant therapy. However, adjuvant therapy status seemed not to have affected DFS in patients with CD133+ as well as CD133- tumors.

Our finding on the one hand questions the non-response to chemotherapy theory and on the other hand asks for further elucidation of the precise prognostic role of CD133 as an important prognostic factor for considering adjuvant therapy in stage II and III colon cancer. Future cohort studies with more number of patients in the two groups

according to adjuvant therapy may further enlighten this finding.

In the results of the CRC cell lines study, the increased mRNA expression of *ABCG2* and *AKT1* after CD133 siRNA transfection further lends support to our hypothesis that CD133 negative rather than CD133 positive colorectal cancer cells might be resistant to adjuvant therapy and that the CD133 positive tumors may benefit from adjuvant therapy. The *ABCG2* protein also called breast cancer resistance protein (BCRP) is involved in the cellular efflux of biologic molecules.[83] In vitro studies indicate that ABCG2 is able to potentially cause multidrug resistance in several malignancies via actively exporting various drugs across the plasma membrane and it was also shown that increased expression and the activity of *ABCG2* are associated with cancer stem-like phenotypes.[84] However, *ABCG2* expression was not concordant with that of CD133, which questions the role of CD133 as a CSC marker. Moreover, the PI3K/PTEN/Akt/mTOR pathway has been implicated as critical player in different aspects of cell growth and malignant transformation.[85] The serine/threonine kinase Akt was also shown to positively regulate *ABCG2* and thereby plays an important role in multidrug resistance.

Survivin is a member of the inhibitor of apoptosis (IAP) family that functions to inhibit caspase activation and by that leads to negative regulation of apoptosis or programmed cell death. *Survivin* is also a direct target gene of the Wnt pathway and is upregulated by *β-catenin*.[86] Tumors that over-express *Survivin* usually have a poor prognosis and are associated with resistance to therapy.[87] The result of *Survivin* in DLD-1 and Caco-2 cells in our study was not concordant and should be cautiously interpreted. On the one hand, *Survivin* is mostly expressed in proliferating cells. Thus, the concordant high expression of CD133 and *Survivin* mRNA and decreased expression of *Survivin* mRNA following CD133 siRNA transfection is in line with the notion that adjuvant therapy works well on rapidly proliferating subset of cancer cells rather than on slowly growing population of tumor cells. Meanwhile, *Survivin* has anti-apoptotic function for which it should not be ignored that CD133 expression might give shelter to cancer cells from apoptosis. Further studies focusing on the correlation of CD133 and *Survivin* seems necessary to clarify this issue.

Beta-catenin which is encoded by *CTNNB1* gene is a dual-function protein that regulates the coordination of cell–cell adhesion and gene

transcription. It is associated with several malignancies and thus considered an oncogene.[88] The regulation of CD133 by HDAC6 was shown to promote *β-catenin* and thereby suppress cancer cell differentiation and maintaining the stem-cell like phenotype of CSCs.[89] It was also suggested that inhibiting *β-catenin* signaling pathway or blocking CD133 may prove a helpful mean for targeting certain cancer progenitor cells. Our results indicate that CD133 knock-down causes decrease in the mRNA expression level of *β-catenin*, supporting the correlation between CD133 and *β-catenin*. However, since *Wnt/β-catenin* pathway is involved in the proliferation of stem cell and cancer stem cell and negatively enhances the differentiation, higher expression of *β-catenin* may also mean that its expression is observed in rapidly proliferating cancer cells which in that sense make them good targets for adjuvant therapy. However, this phenomenon is waiting for comprehensive analysis for valid conclusion.

Taken together, we suggest that CD133 expression might prove helpful in determining the groups of patients who will benefit from adjuvant therapy leading to their longer overall survival.

In NECs, the analysis of correlation between CD133 and survival came short of demonstrating significant difference of overall survival between the CD133 (+) and CD133 (−) groups.

However, the survival analysis in NEC patients in our study has few short-comings. First, small number of study subjects in this study had available follow up data. Secondly, the tumor locations in these patients were different. And at last, the patients had different T-stage. Therefore, a study with large number of subjects in different categories is needed to elucidate the prognostic significance of CD133 expression.

The survival analysis of patients with NENs especially the well-differentiated NETs (G1 and G2) is associated with certain difficulties due to different reasons. Firstly, until recent past, NET G1 and NET G2 (previously called carcinoid tumor) were not included in the cancer registry.[46] The second reason is the indolent course of the NETs, which require long follow up. The third reason is lack of universal acceptance on the nomenclature of NETs and for staging of disease[90], which in turn causes difficulty in evaluating data on a cohort level to specify the

prognostic significance of NENs, specially the low grade tumors. In this study, the group of patients with NECs (n = 23) had available survival data obtained from cancer registry. Although it was previously demonstrated that small cell carcinoma cell lines of lung had a subpopulation that were positive for CD133 and these cells showed stem cell like features and chemoresistence[54-55], there is no available data in the literature so far on human subjects to show the prognostic significance of CD133 expression in NENs. Our study is the first to shows that patients with NECs do not have significantly different prognosis based on their CD133 IHC expression status.

Two things however need to be mentioned here. Firstly, the CD133 expression is not limited to the GEP-NETs with high histologic grade and since it is evident that irrespective of CD133 expression, NET G1 and G2 have a far better prognosis than the NECs (G3) and MANECs[46], therefore the interpretation of the prognostic role of CD133 as a CSC marker seems questionable in GEP-NETs.

Similarly it was recently shown that there is no significant difference in the sphere-forming efficiency between the CD133 (+) and

CD133 (−) groups in the small cell lung cancer cell line H446.[91] Moreover, Kubo et al have demonstrated that among the two groups of CD133(−) and CD133 (+) small cell cancer cell line SBC-7, the CD133(−) group had more tumorigenic potential than CD133 (+) group, and thereby they referred to CD133 as an "inadequate" marker for CSCs.[55] Secondly, as we did not observe the CD133 IHC expression in non-neoplastic neuroendocrine cells in any of the organs in our study, it is of great interest to further study the role and prognostic significance of this molecule in the development of NENs.

CONCLUSION

In conclusion, CD133 is frequently expressed in adenocarcinoma as well as NENs in organs of digestive system compared to non-neoplastic epithelial cells. CD133 expression in CRCs may be regulated by promoter methylation. CD133 IHC expression notifies a better prognosis in stage II and III CRC patients who undergo adjuvant therapy. However CD133 IHC expression is not an independent prognostic factor in patients with stage II and III CRC. Increased mRNA expression of genes related to chemoresistance (*ABCG2* and *AKT1*) after CD133 siRNA transfection in DLD-1 and Caco-2 cells lends more support to our hypothesis that CD133 negative tumors may not benefit from adjuvant therapy.

Unprecedentedly, we demonstrated the expression of CD133 in both well and poorly differentiated GEP-NETs. Although the IHC expression for CD133 was not associated with patients' survival in NECs in our study, the CD133 immunohistochemical expression observed only in tumors (not in normal) of neuroendocrine system is an impelling finding that asks for further exploration.

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국문초록

소화기계에서 암 줄기세포 표지자, CD133 의 발현 및 조절 기전

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연구 배경 : CD133 은 세포막 당단백질로써 소화기계 암을 포함한 여러 악성종양의 암 줄기세포 (CSC) 표지자로 알려져 있다. 종양세포에서 CSC 표지자의 발현은 암의 진행, 전이 또는 항암-방사선 치료에 저항하는 능력과 연관이 있는

것으로 보고되고 있으나 소화기계 암의 대표적인 CSC 표지자인 CD133 발현의 역할과 조절 기전에 대해 많은 보고가 있으나 아직 논란이 있다. **연구 목적** : 소화기계 암에서 암 줄기 세포 표지자 CD133 발현의 역할을 이해하기 위해 첫째, 소화기계의 종양과 비종양성 상피에서 부위별로 CD133 발현의 양상과 빈도를 확인하고 둘째, 발현이 조절되는 기전을 알기 위하여 대장의 선암을 대상으로 프로모터 메틸화 수준과 CD133 발현의 상관 관계를 평가하며 셋째, 대장의 선암과 신경내분비 세포종양을 대상으로 예후인자로서 CD133 발현의 의의를 검사하고자 한다. 마지막으로 대장암 세포 주를 이용하여 CD133 발현의 소실이 세포의 신호전달체계에 어떤 영향을 미치는지를 확인하고 임상적 유의성과 비교함으로써 CD133 발현의 역할을

설명하고자 한다. **연구 방법 :** 임상적 의의를 확인하기 위하여 외과적으로 절제된 480 예의 소화기계 상피종양을 이용하였으며 유전자 발현의 조절을 위해 3 개의 대장암 세포주를 사용하였다. 소화기계 상피종양은 390 예의 선암과 90 예의 신경내분비 종양 (gastroenteropancreatic neuroendocrine tumors, GEP-NETs)으로서 선암은 발생 부위에 따라 위 107 예, 대장 271 예, 췌장 12 예 였다. GEP-NETs 는 위 15, 소장 7, 결장 11, 직장 41, 담낭 4, 간 10, 췌장 2 였다. CD133 발현의 양상과 빈도를 검사하기 위해 수술로 절제된 소화기 암 480 예의 파리핀 블록을 이용하여 면역조직화학염색을 시행하였으며 발현의 정도를 반정량적으로 구분한 후 10% 이상의 발현이 있는 경우 양성으로 판정하였다. 이 검체 중 신선 조직이 보관된 75 예를

대상으로 mRNA 를 정량적으로 분석하였으며 프로모터 메틸화 정도는 대장암 중 171 예의 파라핀에 포매된 조직을 이용하여 정량적으로 분석하여 CD133 발현과 비교하였다. 예후와의 연관성을 평가하기 위하여 면역염색을 시행 한 검체 중 추적 관찰 정보 사용이 가능한 271 예의 대장암과 23 예의 신경내분비 암종을 대상으로 환자의 나이, 성별, 종양의 위치, 분화등급, 침윤 깊이와 림프절 전이 등과 비교하였다. 수술 전 항암치료를 받은 환자는 제외 하였으며 Kaplan-Meier 생존 분석을 사용하였다. DLD-1 과 Caco-2 대장암 세포주를 대상으로 siRNA transfection 을 이용하여 CD133 발현을 억제한 후 항암제 내성과 연관 있는 유전자인 *ABCG2* 과 *AKT1* mRNA 의 발현의 변화와 함께 세포자멸사와 연관 있는 *Survivin*, 세포증식과 연관 있는 *β-catenin* mRNA 발현의

변화를 정량적으로 평가하여 비교 하였다. **연구 결과 :** 소화기계의 비 종양성 점막에서 CD133 의 발현은 매우 드물었으며 위의 유문선과 십이지장 브루너선, 결장의 선 세포 중 극소수, 췌장의 중심선방세포, 간의 담관 세포에서 세포막의 내강면을 따라 발현되었다. 췌장의 섬 세포를 비롯한 소화기계에 산재한 신경 내분비 세포에서는 전혀 발현되지 않았다. 종양 조직에서 CD133 발현 양상은 선암과 GEP-NETs 간에 차이를 보였다. 선암의 경우 대부분 선구조의 내강 면을 따라 종양 세포의 세포막에서 발현되었고 소수의 저분화 선암에서 세포질에서 발현이 관찰되었다. 이에 반해 GEP-NETs 는 대부분 종양세포의 세포질에서 발현이 되었다. 발현 빈도를 보면 선암은 전체 예 중 35.7 % (위암 34 %, 대장암 48 %, 췌장암 25 %)에서 양성이었고 GEP-NETs 은

33 %에서 양성이었다. CD133 면역염색발현은 mRNA 발현 수준과 유의한 상관관계를 보였다(P=0.0257). 프로모터의 메틸화 수준은 CD133 면역염색발현 (p<0.0001) 및 mRNA 발현과 (P=0.11) 역상관 관계를 보였다. 대장의 선암을 대상으로 한 생존분석에서 CD133 발현은 환자의 연령, 성별, 분화도, 침입 깊이와 림프절 전이, 및 생존율(P=0.9689) 과 유의한 상관관계가 없었다. 마찬가지로, GEP-NETS 에서도 CD133 발현과 종양의 등급, 부위 및 신경 내분비세포 표지자 (chromogranin-A 와 synaptophysin)의 발현 및 생존율(P=0.97)과 상관관계가 없었다. 그러나 흥미로운 것은 대장암의 경우 CD133 양성인 군에서 수술 후 항암치료를 받은 경우 치료를 받지 않은 환자에 비해 생존율이 유의하게 좋았다 (p<0.0001, HR =0.125, 95% CI 0.052-0.299). 반면,

CD133 음성인 환자는 항암치료에 따른 생존율의 차이를 보이지 않았다 (p=0.055, HR=0.500, 95% CI 0.247-1.015). 대장암 세포주에 siRNA 를 이용하여 CD133 발현을 억제시킨 경우 대조 군에 비해 항암제 내성과 연관이 있는 *ABCG2* 및 *AKT1* mRNA 는 증가하였고 세포증식과 연관이 있는 *β-catenin* mRNA 의 발현은 감소하였다. 그러나 세포사멸과 연관이 있는 *Survivin* 의 경우 두 세포주간에 서로 다른 결과를 보였다. **결론 :** CD133 은 소화기계 비종양성 상피에서는 매우 드물게 발현이 되지만 선암 뿐 아니라 신경내분비종양에서도 발현이 뚜렷하게 증가하여 부위와 조직 형에 관계없이 소화기계 암종과 연관이 있음을 알 수 있었다. 본 연구를 통해 신경내분비종양에서 CD133 가 발현됨을 처음으로 기술하였다. 또한 대장암에서 CD133 의 발현은 프로모터

메틸화에 의해 역방향으로 조절되는 것을 확인하였다. CD133 발현이 대장암에서 독립적인 예후 인자는 아니지만, 수술 후 항암제 치료의 효과를 예측하는 데 도움이 될 수 있음을 확인하였다. 또한 현재까지 알려진 것과 달리 본 연구에서는 CD133 발현이 항암제 내성을 억제하는 것과 연관이 있을 가능성을 시사한다고 생각한다. DLD-1 과 Caco-2 대장암 세포주를 이용한 연구에서 CD133 의 발현이 항암제 내성과 연관이 있는 *ABCG2* 및 *AKT1* mRNA 의 발현의 조절을 통하여 항암제 내성에 관여한다고 생각한다.

핵심 단어: 암 줄기세포 표지자, CD133 단백질, 소화기계 종양, 선암, 신경 내분비 종양, 예후, 항암치료

PUBLICATION LIST

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