

## RESEARCH ARTICLE

# Increased Expression of P2RY2, CD248 and EphB1 in Gastric Cancers from Chilean Patients

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### Abstract

**Background:** Gastric cancer (GC) ranks as one of the major causes of mortality due to cancer worldwide. In Chile, it is currently the leading cause of cancer death. Identification of novel molecular markers that may help to improve disease diagnosis at early stages is imperative. **Materials and Methods:** Using whole-genome DNA microarrays we determined differential mRNA levels in fresh human GC samples compared to adjacent healthy mucosa from the same patients. Genes significantly overexpressed in GC were validated by RT-PCR in a group of 14 GC cases. **Results:** The genes CD248, NSD1, RAB17, ABCG8, Ephb1 and P2RY2 were detected as the top overexpressed in GC biopsies. P2RY2, Ephb1 and CD248 showed the best sensitivity for GC detection with values of 92.9%, 85.7% and 64.3% ( $p < 0.05$ ), respectively. Specificity was 85.7%, 71.4% and 71.4% ( $p < 0.05$ ), for each respectively.

**Keywords:** Gastric cancer - microarray - molecular markers - P2RY2 - EphB1 - CD248

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### Introduction

Gastric cancer (GC) is one of the leading causes of cancer death worldwide, with 989,600 new cases and 738,000 estimated deaths in 2008 (Wang et al., 2012). While in most countries the mortality rate is declining, in Chile the mortality rate has remained relatively stable in the last 3 decades (Arancibia et al., 2009). Furthermore, GC currently ranks as the leading cause of cancer death in Chile, with a crude mortality rate of 20/100,000 inhabitants over the last 10 years (25.1/100,000 males and 13.2/100,000 females) (Heise et al., 2009). In 2002, 3,115 deaths were secondary to GC in Chile (Arancibia et al., 2009).

GC affects men preferably, with a male:female ratio of 2.6:1. The highest incidence is observed between 60-80 years old. However, if only early stage patients are considered, the average age drops to 40-45 years old (MINSAL, 2010). Importantly, the GC outcome is directly related to the stage observed at the time of diagnostic confirmation. It has been estimated that about half of Chilean patients already have metastatic nodules or neighboring organ involvement by diagnosis and most patients subjected to resection are in advanced stages of the disease (MINSAL, 2010). Therefore, GC screening tools are urgently needed.

Endoscopy of the esophagus, stomach and duodenum with biopsy is the standard method for GC diagnosis. This technique is very sensitive when performed by an experienced specialist, and can detect early-stage lesions. However, the progress of GC is adversely affected in patients seeking healthcare at the Chilean health public system since they are subjected to waiting times of about 3-months in average (unpublished data). Moreover, patients with early GC type specific symptoms manifest dyspeptic and alarm symptoms (anemia, dysphagia, weight loss) that are indistinguishable from patients with benign disease. On the other hand, the majority of patients with advanced GC show symptoms including recurrent abdominal pain, anemia, weight loss, vomiting and anorexia (MINSAL, 2010).

With this background, we perceive the need for non-invasive methods for GC early detection aiming to reduce GC mortality. Searching for early detection GC biomarkers, including proteins, can be a powerful tool for control and reduce the mortality rate due to GC (He and Zhang, 2013). Various genes involved in both cell cycle control and in cell adhesion, undergo altered expression due to cancer, and can be detected by molecular techniques in various tissues and biological fluids (Yasui et al., 2011). In recent years, many genes have been evaluated as biomarkers, such as ADAM17 (Zhang et al., 2011),

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MMP2, MMP9 and MMP11 (Sampieri et al., 2010; Zhao et al., 2010), Survivin and CK19 (Bertazza et al., 2009), Vimentin (Iwatsuki et al., 2010), CXCR4 (Ingold et al., 2010), ING5 (Xing et al., 2010), Stanniocalcin2 (Yokobori et al., 2010) among others. In this regard, MMP11 has been shown overexpressed in 72.5% of gastric tumor tissue respect of healthy mucosa (Zhao et al., 2010) while CK-19 and Survivin showed overexpression in 98.6% and 97.1% respectively, in GC samples (Bertazza et al., 2009). Similarly, 77.7% of gastric tumors overexpressed Stanniocalcin-2 respect to the normal mucosa of healthy patients (Yokobori et al., 2010). Even CEA and CA 19-9 may be of assistance (Sisik et al., 2013), as well as microRNAs (Ma et al., 2013). Clearly, the possibility of measuring the level of expression of certain genes in a biological sample obtained from a patient, gives the possibility of developing a cancer screening test through molecular markers.

In this work, we identified by DNA-microarrays, a set of genes that displayed altered expression in GC endoscopic biopsies. Then, selected transcripts were validated by RT-PCR in tissue samples from additional patients with GC. We propose these genes as possible biomarkers of GC.

## Materials and Methods

### Tissue samples

Samples of gastric mucosa from 14 patients with histopathological diagnosis of GC who underwent surgery were analyzed. For each patient, surgical specimens were a biopsy of the tumor and a biopsy of the healthy mucosa adjacent to it. Patients were aged 55 years and above. Patients participated voluntarily in the study by signing an informed consent. The present protocol was approved by the Bioethical Committee of Health Service of Coquimbo, Chile.

### RNA isolation and RT-PCR

RNA was isolated from approximately 50mg of biopsy samples using the Absolutely RNA Miniprep Kit (Stratagene®). For the 14 patients, semiquantitative RT-PCR assays were conducted in cDNAs obtained from 1µg of RNA of each normal/tumor pair using the Improm II reverse transcriptase (Promega®) according to manufacturer's instructions. For DNA microarray hybridizations, cDNAs were synthesized from 2µg of total RNA of 5 selected normal/tumor pairs, using the SuperScript II (Invitrogen®) reverse transcriptase.

### cDNA labeling and DNA microarray hybridization

cDNAs obtained as described above were fluorescently labeled with the Label IT® µArray™ Cy™ 3/Cy™ 5 Labeling Kit (Mirus™) following instructions of manufacturer. Tumor samples were labeled with Cy3 while normal samples were labeled with Cy5. After clean up reaction mixtures, labeled cDNAs were mixed with an equal volume of a hybridization solution containing 50% formamide, 10µg of fish sperm DNA, 0.1% SDS and 10X SSC buffer. The total volume of 70 ul was deposited on HEEBO (Human exonic evidence based)

-70mer oligonucleotide microarrays (48K), covered with a LifterSlip (Thermo Scientific), placed inside hybridization chambers(ArrayIt) and incubated at 42°C for 18 hrs in a water bath. Microarrays were scanned in a ScanArray Lite instrument (Perkin-Elmer). Two-channel images were saved in TIFF format and then extracted with the GenePix Pro 5 software. GPR files were normalized by print-tip loess with the DNMAID tool (<http://dnmaid.bioinfo.cnio.es/>), data values filtered out 0.5 SD cut-off and subjected to two "one class" tests: a SAM (significance analysis of microarray) and a limma (linear models for microarray data) using the MeV v4.7 tool (<http://mev.tm4.org/>).

### Semiquantitative RT-PCR validation of selected genes

The expression level of six genes selected for validation was determined by RT-PCR. The genes selected by microarray were P2RY2, CD248, NSD1, RAB17, ABCG8 and EphB1. B2M was used as housekeeping gene. Briefly, 2µl of cDNA were incubated in presence of 1.5µl of 25mM MgCl<sub>2</sub>; 0.5µl of 10mM dNTPs, 2µl of each primer (10µM), 0.2µl Platinum Taq (Invitrogen) in a final volume 25µl. The amplification conditions are shown in Table 1. Once validated, 3 of these genes were selected as molecular markers candidates. At this point, the PCR protocol was reduced in number of cycles from 35 to 30, to more accurately observe changes in expression levels across cancer samples. In this way we corrected the cut-off parameters in the selection of healthy/diseased aiming to increase the sensitivity of the technique.

PCR amplification was visualized by 1.2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Expression levels were analyzed semiquantitatively using the Quantity One software (Biorad®).

### Data analysis

Difference in average expression of the genes was addressed with the t-test for paired data student (non-independent samples), hypothesis testing as expected unilateral increased gene expression in tumor tissue. Statistical significance was set at a p=0.05. These analyses, as well as estimates of the sensitivity and specificity of the RT-PCR technique for GC detection, were performed with XLSTAT Version 2011.2.06.

## Results

### Microarray experiments

Tumor/normal pair samples were compared in 2-color cohybridization DNA microarray experiments using a whole genome platform. After statistical analyses, a total of 446 probes corresponding to 352 unique genes showed significant altered expression in 5 patients evaluated, affected by GC (Figure 1 and 2). Of these, 86 genes were up-regulated and 266 genes were down-regulated. The top 6 overexpressed genes (tumor over normal) were selected for further validation (Table 2).

### Validation of selected genes using RT-PCR

Altered expression of P2RY2, CD248, NSD1, RAB17, ABCG8 and EphB1 detected by DNA-microarray analysis

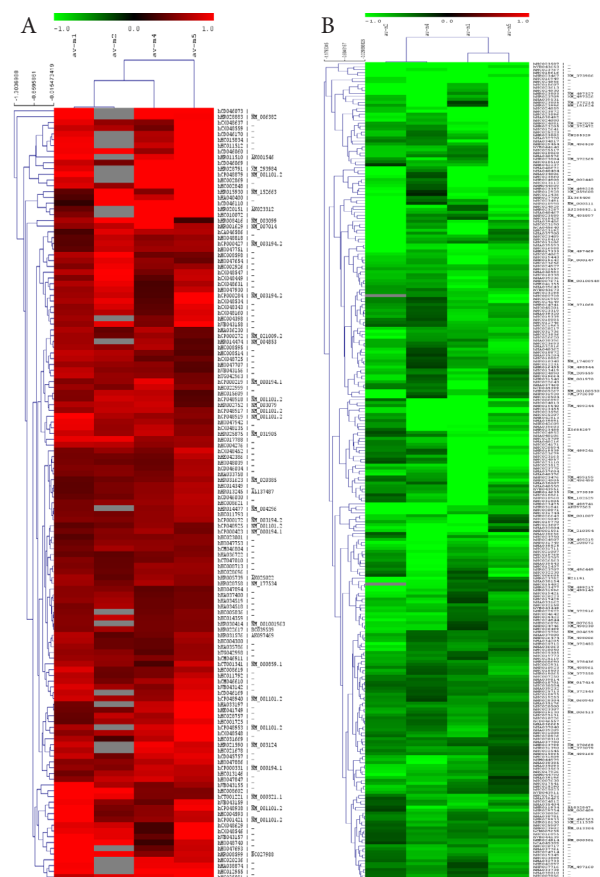
**Table 1. Primer Sequences and PCR Conditions**

Genes	Sequence	PCR product size (pb)	PCR Conditions
P2RY2	FP 5'-CCACTGGCCCTTCAGCACGG-3' RP 5'-GCGGGTGACGTGGAATGGCA-3'	505	95°C5 min (1 Cycle); 95°C1 min, 66°1 min 72°C1 min (35 Cycles); 72°C10 min
CD248	FP 5'TTTGGCTTCGAGGGCGCCTG'3 RP 5'CACACTGCTGCTCGCACGGA'3	371	95°C5 min (1 Cycle); 95°C1 min, 66°1 min 72°C1 min (35 Cycles); 72°C10 min
NSD1	5'CAAGAGACGCCCATGGTGGC3' 5'TCTGGCTCGAGATTAGCGCA3'	508	95°C5 min (1 Cycle); 95°C1 min, 62°1 min 72°C1 min (35 Cycles); 72°C10 min
RAB17	5'ACCACCAGGTGTCTGGAGGTG3' 5'GGAACAGGCACAGGCATCGG3'	403	95°C5 min (1 Cycle); 95°C1 min, 65°1 min 72°C1 min (35 Cycles); 72°C10 min
ABCG8	5'ATGTACGTGCGGGGTTGTC3' 5'GGGGTGCCAGACGTCATCAG3'	251	95°C5 min (1 Cycle); 95°C1 min, 64°1 min 72°C1 min (35 Cycles); 72°C10 min
EPHB1	5'AATGGCATCATCCTGGACTA3' 5'TCAATCTCCTTGCAAATCTC3'	463	95°C5 min (1 Cycle); 95°C1 min, 57°1 min 72°C1 min (35 Cycles); 72°C10 min
Control			
B2M	5'ACCCCACTGAAAAAGATGA'3 5'CTCAGATACATAAACATGG'3	286	95°C5 min (1 Cycle); 95°C1 min, 55°1 min 72°C1 min (35 Cycles); 72°C10 min

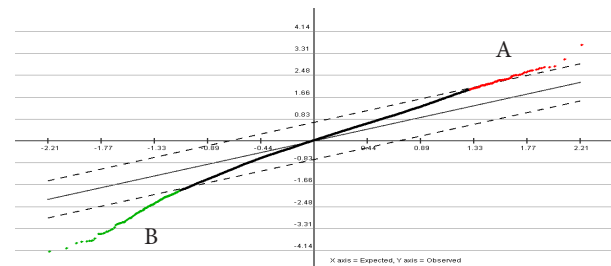
**Table 2. Genes with Highest Overexpression in GC**

Symbol, description <sup>a</sup>	Relative expression (log <sub>2</sub> average) <sup>b</sup>	Biological role
P2RY2, purinergic receptor P2Y, G-protein coupled	1.09	G-protein coupled to receptor
CD248, CD248 molecule, endosialin	0.87	Proliferation
NSD1, nuclear receptor binding SET domain protein 1	0.86	Epigenetic control
RAB17, member RAS oncogene family	0.84	Epithelial GTPase
ABCG8, ATP-binding cassette, sub-family G, member 8	0.82	Cholesterol Transport
EPHB1, Ras and Rab interactor 2	0.79	Tyrosin Kinase Receptor

\* <sup>a</sup>Microarray data (~18,000 oligonucleotides in at least 4 of 5 samples) were normalized by loess and then subjected to statistical tests (see Methods) for one class. 446 probes were statistically significant (p < 0.001). The 6 genes extremely upregulated are shown; <sup>b</sup> The ratios of relative expression corresponds to the average for 5 patients of the transcript levels in the tumor biopsy above the normal biopsy, converted to log base 2



**Figure 1. Summary Heatmap of DNA Microarray Data Showing 128 Overexpressed Genes (A) and 318 Repressed (B) That Showed Statistical Significance in at Least 4 from 5 GC Samples**



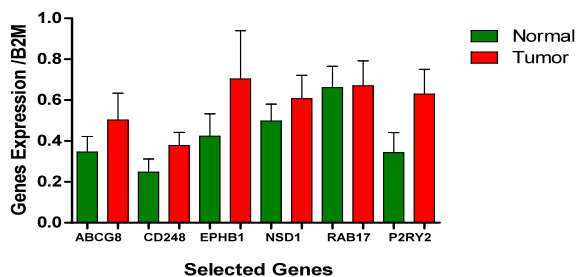
**Figure 2. Significance Analysis of Microarrays (SAM) Plot of GC Expression Profiles.** RNA from tumor tissue was compared to healthy adjacent mucosa. Expected differentially expressed genes are in the X axis, while observed differentially expressed genes are in the Y axis. Genes repressed (A) are located in the lower left quadrant of the diagram and overexpressed genes (B) in the right upper quadrant. Genes without significant altered expression are shown as a black line. Parallel dotted lines correspond to the delta values cut-off. Solid line indicates the equal value of observed and expected genes differentially expressed

was validated using RT-PCR in advanced gastric tumor tissue of 10 patients of the region of Coquimbo (Chile). As a control, we used healthy mucosa adjacent to the lesion for the same patients. The expression of each gene was measured with the Quantity One<sup>®</sup> program (BioRad<sup>®</sup>) and standardized against the housekeeping gene B2M. According to this sensitivity and specificity of each gene, predictive values for GC were calculated. Results are shown in Figure 3 and Table 3.

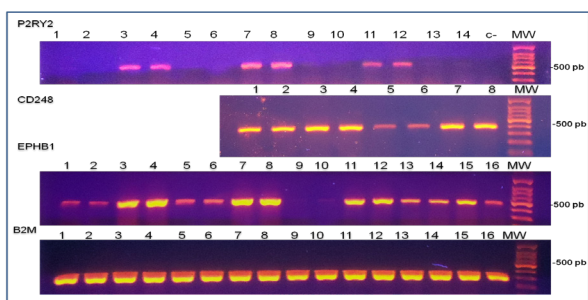
As shown in Figure 3, semiquantitative RT-PCR was able to detect overexpression of all except one of the genes evaluated in tumor tissue compared to the adjacent

**Table 3. Sensitivity and Specificity of Selected Transcripts as GC Predictors in Tumor Tissue from 10 GC Patients**

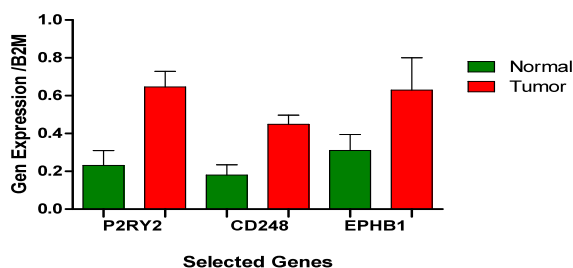
Marker	P2RY2	CD248	NSD1	RAB17	ABCG8	EPHB1
Sensitivity%	80	70	55	55	60	80
Specificity%	80	60	55	55	60	50



**Figure 3. Expression mRNA Levels of ABCG8, CD248, EphB1, NSD1, RAB17 and P2RY2 Genes Relative to the Housekeeping B2M Gene in 10 Patients with GC.** CD248, EphB1 and P2RY2 showed significant differences (\*) in their expression when comparing normal with tumor tissue with  $p < 0.05$



**Figure 4. 1.2% Agarose Gel Electrophoresis Analysis Showing Expression of P2RY2, CD248 and EphB1 Genes in Healthy Mucosa (Lanes 1-2, 5-6, 9-10, 13-14) and Gastric Tumor (Lanes 3-4, 7-8, 11-12, 15-16) of Patients with GC.** Results are shown in duplicate for each patient. Normalization was done against the housekeeping B2M gene. In all cases, mRNA levels of candidate genes in tumor tissue are higher than healthy mucosa



**Figure 5. Relative Expression of Genes P2RY2, CD248 and EphB1, in Relation to the Housekeeping Gene B2M in Tumor and Normal Gastric Mucosa Obtained from 14 Patients with GC**

healthy mucosa. Overexpression of NSD1 and ABCG8 was not significant, similar to RAB17 downregulation in tumor tissue relative to healthy tissue. These 3 genes were those with lesser sensitivity in GC detection, so they were discarded as candidates to be GC biomarkers.

**Table 4. Sensitivity and Specificity of the Use of P2RY2, CD248 and EPHB1 as Molecular Markers for Detect GC Using RT-PCR in Gastric Mucosa Samples from 14 Patients**

Marker	Sensitivity	Specificity
P2RY2	92.9%	85.7%
CD248	85.7%	71.4%
EPHB1	64.3%	71.4%

Conversely, the genes P2RY2, CD248 and EphB1 showed higher levels of overexpression in tumor tissue and better sensitivity in GC detection. Therefore, they were selected for further study. These three molecular markers were evaluated by RT-PCR in gastric tumor tissue and adjacent healthy mucosa of a total of 14 patients with advanced GC including the 10 previous patients.

Again, these genes showed higher overexpression in the tumor respect to the healthy mucosa. P2RY2, CD248 and EphB1 genes showed 2.79, 2.48 and 2.03-fold overexpression respectively in the tumor compared to healthy mucosa. The P2RY2 gene sensitivity as a biomarker for predicting GC was 90%, while for CD248 and EphB1, the sensitivity was 85.7% and 64.3% respectively.

The results are shown in Figure 4 and 5, and Table 4.

## Discussion

GC is currently one of the leading causes of cancer death in Chile and the world, which is why efforts should be made in the early diagnosis of this malignancy. Using whole genome DNA-microarrays, we detected altered expression of 352 genes in tumor tissues from a group of patients with advanced GC. Of these, we selected the top 6 overexpressed genes for validation by conventional RT-PCR. These genes were P2RY2 (purinergic receptor P2Y, G-protein coupled), CD248 (endosialin), NSD1 (nuclear receptor binding SET domain protein 1), RAB17 (member RAS oncogene family), ABCG8 (ATP-binding cassette, sub-family G, member 8) and EphB1 (Ras and Rab interactor 2). After the validation process, we determined that 3 of them had high levels of sensitivity and specificity for GC detection in tissue samples. P2RY2 presented a sensitivity of 92.9% and a specificity of 85.7% for the disease detection. P2RY2 belongs to the family of purinergic receptors coupled to G protein, of which there are five subtypes expressed in human, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 (Faria et al., 2012). P2Y2 receptor recognizes ATP and UTP ligands, and bind G-protein by phospholipase C (PLC) activation and mobilizing intracellular  $Ca^{2+}$  (Li et al., 2011). This receptor subtype is involved in proliferation of certain tumors including lung (Schumacher et al., 2013), pancreas (Choi et al., 2013), prostate (Li et al., 2013) and breast (Li et al., 2011). In contrast, in colorectal cancer, this receptor blocks cell proliferation (Küntzli et al., 2011). To date, there are no data regarding the effect of P2RY2 receptor in GC. Therefore, based on our results, we suggest that the role of P2RY2 in gastric tumors is to induce cell proliferation, because its increased expression (2.79 fold) in the tumor

over normal mucosa.

Another gene, CD248, resulted with a sensitivity of 85.7% and a specificity of 71.4% for the GC detection. This gene, also known as TEM1, codes for endosialin, a type I surface glycoprotein with a predicted 80.9 kDa molecular mass of 757 amino acids. CD248 interacts with extracellular matrix (ECM) proteins and thereby participates in cell adhesion, migration and regulation of inflammation (Maia et al., 2011; Valdez et al., 2012). It has been demonstrated that this gene is commonly overexpressed in colorectal cancer, breast cancer, brain tumors, melanoma, and squamous cell carcinoma, particularly in angiogenic vasculature pericytes and adjacent fibroblasts (Bagley et al., 2008b; Simonavicius et al., 2008; Zhang et al., 2011). The mechanism of CD248 action in cancer seems to be related to the extracellular protein domain that interacts with ECM proteins thus facilitating the interaction with matrix metalloproteinases like MMP-9, cell migration and metastasis. Moreover, it has been described that the highly conserved cytoplasmic domain of CD248 is able to mediate regulatory signals for stromal fibroblasts (Maia et al., 2011). Transgenic mice with this domain deleted, showed reduced tumor growth, invasiveness and metastasis (Naylor et al., 2012). There are no previous reports for CD248 overexpression associated with GC.

Finally, this study found that the EphB1 gene showed a 2-fold overexpression in the tumor relative to adjacent healthy mucosa, and presented a sensitivity of 64.3% and a specificity of 71.4% for GC detection. EphB1 is a member of the EPH family of tyrosine kinases receptors, which are categorized into two subclasses according to their structure and the affinity for its ligand Ephrin. Class A Eph receptors (EphA) bind to ligands anchored to glycosylphosphatidylinositol, and class B Eph receptors (EphB) bind to ligands anchored to transmembrane domains (Ephrin B) (Kim et al., 2011). EPH family receptors play a critical role in embryonic development and angiogenesis, and are also crucial in guiding neuronal axon guidance and morphogenesis of dendritic spines and synapses (Pesti et al., 2012; Bouche et al., 2013).

Altered expression of Eph receptors has been associated to malignant transformation, metastasis, and poor prognosis of cancer. In particular, a study revealed that EphB1 mRNA was overexpressed in 68.9% of a group of patients with GC, while it was suppressed in a 14.8% of them. However the protein expression showed a different pattern, with a 17.2% of patients who overexpress the protein and a 44.8% having suppression of it (Wang et al., 2007). This same group of investigators found a direct relationship between the suppression of the protein and invasion, status and metastasis in GC (Wang et al., 2014).

Apparently, the genes detected in the present study belong to different signaling pathways since there are no reports linking them. However, as mentioned, they are all directly related to the progression of cancer.

Our work is intended to be the basis for future diagnostic studies aimed at using P2RY2, CD248 and EphB1 transcripts as molecular biomarkers of GC. These markers can be measured together or separately, by RT-PCR in gastric tissue samples and be a complement

to the clinical evaluation. In this regard, it has been determined that the combined use of genes by RT-PCR assay significantly improves the sensitivity and specificity of cancer detection (Bernal, 2012). Probably the use of molecular markers is the next step in the clinical diagnosis of cancer for early screening.

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