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Frequencies and expression levels of programmed death ligand 1 (PDL1) in circulating tumor RNA (ctRNA) in various cancer types

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Abstract

Background: Precision medicine and prediction of therapeutic response requires monitoring potential biomarkers before and after treatment. Liquid biopsies provide noninvasive prognostic markers such as circulating tumor DNA and RNA. Circulating tumor RNA (ctRNA) in blood is also used to identify mutations in genes of interest, but additionally, provides information about relative expression levels of important genes. In this study, we analyzed PD-L1 expression in ctRNA isolated from various cancer types. Tumors inhibit antitumor response by modulating the immune checkpoint proteins programmed death ligand 1 (PD-L1) and its cognate receptor PD1.

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The expression of these genes has been implicated in evasion of immune response and resistance to targeted therapies.

Methods: Blood samples were collected from gastric (GC), colorectal (CRC), lung (NSCLC), breast (BC), prostate cancer (PC) patients, and a healthy control group. ctRNA was purified from fractionated plasma, and following reverse transcription, levels of PD-L1 expression were analyzed using qPCR.

Results: PD-L1 expression was detected in the plasma ctRNA of all cancer types at varying frequencies but no PD-L1 mRNA was detected in cancer-free individuals. The frequencies of PD-L1 expression were significantly different among the various cancer types but the median relative PD-L1 expression values were not significantly different. In 12 cases where plasma and tumor tissue were available from the same patients, there was a high degree of concordance between expression of PD-L1 protein in tumor tissues and PD-L1 gene expression in plasma, and both methods were equally predictive of response to nivolumab.

Conclusions: PD-L1 mRNA can be detected and quantitated in ctRNA of cancer patients. These results pave the way for further studies aimed at determining whether monitoring the levels of PD-L1 mRNA in blood can identify patients who are most likely to benefit from the conventional treatment.

Keywords

PD-L1; Circulating tumor DNA; Liquid biopsy; Cancer

1. Background

Past efforts in improving cancer treatment have largely consisted of screening, development of new anti-cancer agents, multi-drug combinations and advances in the radiation therapy. A more recent approach is precision medicine, which takes individual variability into account in order to design personalized treatment strategies [1]. An important goal of precision medicine is to identify molecular markers indicative of therapy selection by analyzing the factors involved in the therapeutic effects and prognosis [2,3]. So far, such information has been obtained by analysis of genes and proteins in cancer tissue biopsies. However, the use of tissue biopsies has many problems, including possible sampling bias and a limited ability to monitor tumor markers in patients during the course of the therapy.

In 1977, Leon et al. discovered that serum ctDNA levels were higher in patients with cancer [4], suggesting that the extra serum DNA in cancer patients originates from the tumor. Subsequent work confirmed this and established that the ctDNA reveals the same information about the patient's genes as that found in the tumor without an invasive tissue biopsy [5−7]. The genetic information from liquid biopsies resides in circulating cancer cells (CTC), circulating tumor DNA (ctDNA), circulating tumor RNA (ctRNA) and exosomes [8]. Cell-free or extracellular RNAs have been detected in many human bodily fluids, including serum/ plasma, saliva, cerebrospinal fluid, synovial fluid, tear fluid, amniotic fluid and urine [9]. For those genes that are expressed, ctRNA contains the same mutational information as does ctDNA, but in addition, can also provide information about the quantitative expression levels of genes of interest.

It is now well established that tumors can inhibit autoimmune antitumor activity by modulating the expression of inhibitory factors and immune checkpoint proteins like programmed death ligand 1 (PD-L1) and its cognate receptor, programmed cell death 1 (PD-1) [10−14]. As an immune suppression mechanism, the expression of PD-L1 is elevated in many types of cancer and is often correlated with poor patient prognosis and predictive of responses to the antibodies against PD-1/PD-L1 [15−17]. Therapies that block this interaction have demonstrated very promising clinical activity in several cancer types [11,12,17,18]. Excluding currently used antibodies for such therapies, inhibition of PD-L1 expression could also have a therapeutic effect. As PD-L1 expression is regulated by mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways, inhibition of these pathways should reduce PD-L1. Indeed, receptor tyrosine kinase inhibitors result in better treatment outcome in lung cancers with high-expression of PD-L1 [17,19,20]. The PD-1 inhibitor, pembrolizumab is now under evaluation in 13 trials across more than 30 types of cancer including bladder, colorectal, gastric, head and neck, melanoma, non-small and small cell lung (NSCLC), renal, pancreatic, prostate, triple negative and estrogen-receptor positive human epidermal growth factor receptor 2 (HER2)-negative breast, gynecologic, and hematologic malignancies [21].

There has to date been no report about using ctRNA to detect PD-L1 expression and there are only a few studies across the various cancers about ctDNA [22]. The purpose of this study was to analyze the frequency and level of PD-L1 expression in ctRNA isolated from various cancer types.

2. Methods

2.1. Patient samples and samples transportation

A total of 760 patient samples were blinded and accessioned by Liquid Genomics, Inc.(later acquired by NantHealth, Inc.) The cancer types comprising the samples included; 44 gastric cancers (GC), 212 colorectal cancers (CRC), 320 non-small cell lung cancers (NSCLC), 24 breast cancers (BC), and 88 prostate cancers (PC). Only cancer types (not histologic subtypes) were known of the samples. All cancer patients had distant metastases and had scheduled chemotherapy. Patient samples were from University of Southern California Norris Comprehensive Cancer Center, Memorial Cancer Institute or University Hospital Essen, West German Cancer Center. This study was approved by the Institutional Review Board of each facility. Ten milliliters of blood were collected in each of two tubes containing a proprietary nucleic acid preservation cocktail and transferred to Liquid Genomics, Inc. as soon as possible. After blind accessioning, all samples proceeded to the isolation process within five days after collection. A two-dimensional bar code was placed on all plasma samples for automatic identification.

2.2. Fractionation of plasma and extraction of ctRNA

Whole blood in 10 mL tubes was centrifuged to fractionate plasma at 16,000 rcf for 20 min ctRNA was extracted from 2 mL of plasma with a proprietary in-house developed protocol especially designed to remove potential contaminating blood cells during the extraction. All

nucleic acids were kept in bar-coded matrix storage tubes. RNA was stored at −80 °C or reverse-transfected to complementary DNA (cDNA) and cDNA was stored at −4 °C.

2.3. The detection of PD-L1 with quantitative real-time PCR

Quantitative real-time PCR was used to detect the expression of PD-L1 in ctRNA. We designed the primers to investigate the expression of PD-L1. Amplification was performed in 10 μL reactions containing 2 μL cDNA, the primers, the probe, and the reaction mix with qPCR and an in-house developed assay. β-actin was used as an internal control. PD-L1 delta-Cts (dCts) were calculated from the PD-L1 Ct value by using b-actin as the positive control dCts were the Ct value of PD-L1 subtracted by the Ct value of β-actin. Next we calculated K using universal human reference (UHR) as a positive control. With using this K, we can reach to the Relative PDL1 Gene Expression.

Relative PD-L1 Gene Expression= (2^-dCT)*K

Relative PD-L1 Gene Expressions were compared across various cancers.

2.4. Digital droplet PCR

Digital PCR was performed on QX200 digital PCR system (BioRad, Hercules, CA). The 96-well PCR plate was prepared with 2×ddPCR Supermix for probes (no dUTP) (Bio-Rad), PD-L1 primer and β-actin VIC primer, and partitioned into a median of ~14,000 droplets per well in QX200 droplet generator according to the manufacturer's instructions. After PCR amplfication, the plate was read on QX200 droplet reader with QuantaSoft v1.7.4.0917 software (Bio-Rad). At least two negative control wells with no DNA were included in every run. The amount of PD-L1 was calculated from the concentration provided by the software.

2.5. Statistics analysis

Statistical analysis was carried out using JMP version 12.2 (SAS Institute Inc., Cary NC). The frequency of expression in cancer types were calculated and compared with each other using analysis of variance (ANOVA). The relative expression levels were compared across cancer type using the Median test. The Chi-Square test compared the normal and control groups. We considered $p < 0.05$ as statistically significant.

3. Result and discussion

An important goal of this study was to demonstrate the feasibility of quantitating PD-L1 gene expressions using ctRNA isolated from blood samples taken from cancer patients as a potential alternative to assaying PD-L1 from solid tumor tissue biopsies. Besides just the detection of PD-L1 expression, which is a prerequisite for the use of PD-L1 directed immunotherapy, we also wanted to set the stage for future studies to determine if the actual quantity of PD-L1 expressed in tumors might provide additional predictive insight into the patient's outcome from therapy with PD-L1 directed agents.

The procedure that was followed in this study to isolate ctRNA is summarized as a flow chart in Fig. 1. Plasma contains both ctRNA as well as ctDNA, but the procedure described here results in isolation of ctRNA that is essentially free of contaminating DNA. In contrast

to ctDNA, ctRNA is relatively unstable in biological fluids presumably due to the presence of ribonucleases, and thus extra precautions are needed when working with ctRNA. To deal with this potential problem, blood drawn in the clinics of origin was immediately placed into storage tubes specially designed to stabilize RNA and to prevent lysis of normal blood cells. However, even then the RNA is not stabilized indefinitely and must be received in the analytical laboratory and processing begun within 7 days. Once RNA is isolated from the plasma, it can be stored at 80 C if necessary, but we usually proceeded with the PCR analysis as soon as possible to avoid any potential effects of freeze-thawing the sample. The isolation-analysis process generally could be completed within 2 days.

For validation purposes, the detection accuracy of the conventional qPCR, which was our method of choice, was assessed by comparing the results of qPCR analysis of PD-L1 from 50 clinical blood samples against those of the Digital Droplet PCR methodology. Among the 50 samples tested, 5 contained insufficient cDNA for analysis and 2 samples were discordant between the two platforms. The remainder of the samples was concordant between the two platforms, corresponding to a specificity of 92.7% and a sensitivity of 96.7% for qPCR. We therefore judged the qPCR platform to be of sufficient accuracy for use in this study. The performances of these two systems also had previously been shown to be very comparable in an EGFR mutation assay [23].

Frequencies, medians and ranges of PD-L1 expression as detected by qPCR in ctRNA isolated from blood samples of patients with the various types of cancer are shown as box plots in Fig. 2. These frequencies were statistically different across the five cancer types ($p < 0.0001$, Fisher's Exact Test). For comparison, we also listed PD-L1 protein expression frequencies independently determined in previous studies by immunohistochemistry of tissue biopsies from the same tumor types (Table 1). The frequency values for NSCLC, breast, gastric and colorectal cancers determined by the two approaches are similar, while those for prostate cancer are somewhat discordant, being lower with qPCR than with IHC. This discrepancy may be due to the relatively small sample sizes in our study and/or to methodological variations in the evaluation and reporting of IHC data, which are often seen as somewhat subjective and semi-quantitative. While variations were noted in median relative PD-L1 expressions among the various cancer types, the differences were not statistically significantly different across the five cancer types ($p = 0.406$, Median Test).

No PD-L1 mRNA was detected in any individuals of the cancer-free control group (0/19). As shown in Table 1, the difference in PD-L1 expression between cancer-free individuals and cancer patients was highly statistically significant by Fischer's Exact Test, not only with the entire group of cancer patients but with each individual cancer as well. This result suggests a possible use of plasma PD-L1 expression as a cancer detection method.

Previous studies of frequency of mutation detection in DNA have reported a high concordance (95%) between ctDNA analysis from plasma and tissue DNA analysis [29,30]. We had available 12 NSCLC tumor tissue samples from patients who had been treated with nivolumab, thus allowing us to perform concordance analysis between PD-L1 protein in tumor tissue and PD-L1 plasma gene expression in the same patients. All 5 of these samples that were positive for PD-L1 by IHC also had various measurable levels of PD-L1 gene

expression (Fig. 3). Of the 7 samples that were negative by IHC, 5 also had undetectable gene expression, while the remaining 2 had low but detectable gene expression. This result indicates a high degree of concordance for PD-L1 analysis between the two methodologies and shows that either one can effectively predict response to nivolumab.

4. Conclusions

PD-L1 mRNA (gene expression) can be detected and quantitated in the ctRNA of cancer patients with frequencies of detection similar to those obtained by previous IHC analysis of solid tumor tissues. Gene expression in plasma predicts response to Nivolumab as effectively as IHC of tumor tissue. These results pave the way for further studies aimed at determining whether monitoring the quantitative levels of PD-L1 mRNA in blood before and during treatment can provide additional predictive value for anti-PD-L1 therapy. Moreover, the lack of PD-L1 mRNA in any of the cancer-free individuals suggests that the presence of PD-L1 mRNA in blood may be a potential marker for detecting the presence of cancer.

Abbreviations

QOL	quality of life
CTC	circulating cancer cells
CtDNA	circulating tumor DNA
CtRNA	circulating tumor RNA
PD-L1	proteins like programmed death ligand 1
PD-1	programmed cell death 1
MAPK	mitogen-activated protein kinase
PI3K	phospho-inositide 3-kinase
HER2	human epidermal growth factor receptor 2
GC	gastric cancer
CRC	colorectal cancer
NSCLC	nonesmall cell lung cancer
BC	breast cancer
PC	prostate cancer
QPCR	quantitative polymerase chain reaction
DCT	delta Ct
ANOVA	analysis of variance

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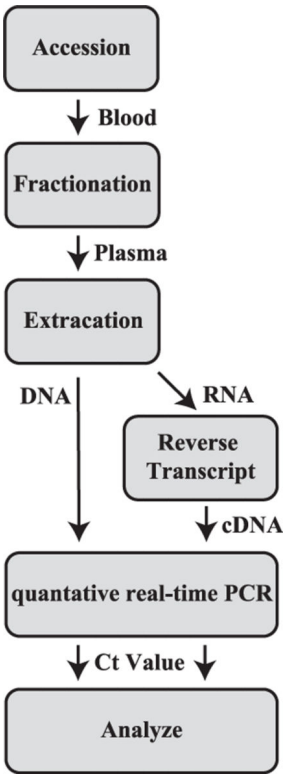

Fig. 1.

Flow diagram of measuring ctDNA and ctRNA. The blood sample was accessioned, fractionated and extracted. After extraction, ctDNA was analyzed with qPCR. ctRNA was reverse-transcripted to cDNA and analyzed with qPCR.

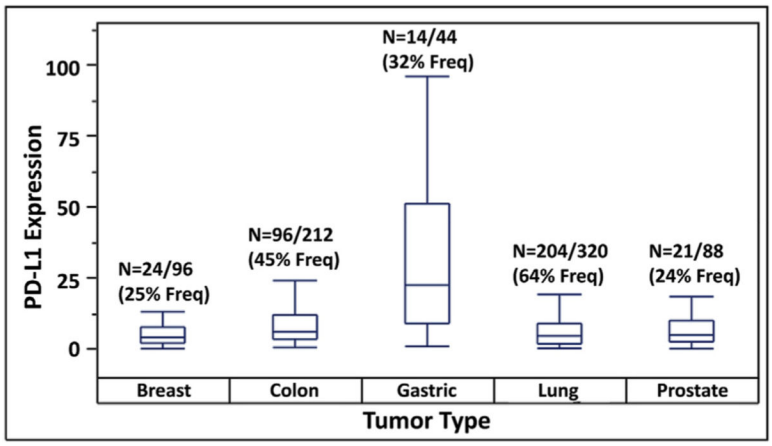

Fig. 2.

Frequencies, medians and ranges of PD-L1 expression determined by analysis of ctRNA isolated from blood samples of cancer patients.

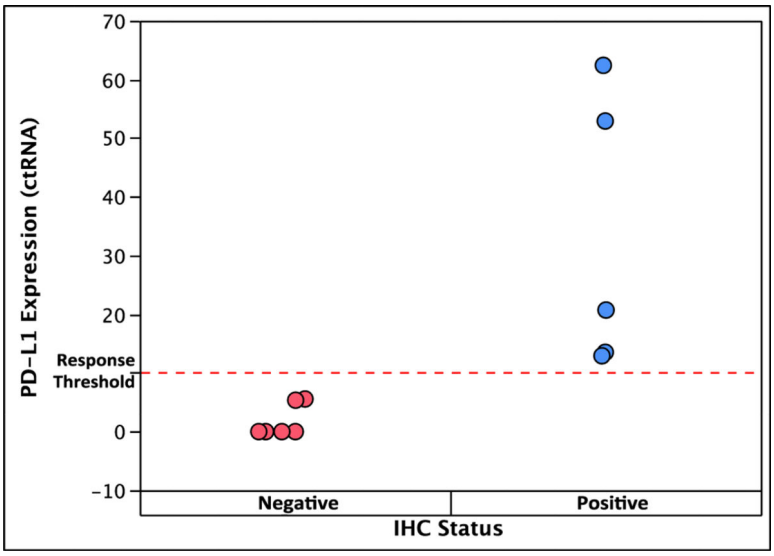

Fig. 3.

Concordance between PD-L1 protein analysis by IHC in tumor tissue and gene expression analysis by PCR in plasma in the same patients and association with nivolumab response. Blue color: response; red color: no response.

Table 1

Detection frequencies and relative values of PD-L1 gene expression in plasma from patients with various cancer types and in cancer free individuals.

Plasma from individuals with	Detection frequency (%)	Detection frequency by IHC (ref)	P values for cancer-healthy difference
No cancer	0.0 (0/19)	—	—
Gastric Ca	31.8 (14/44)	29.6 [24]	0.006
CRC	44.8 (96/212)	25.8 [25]	<0.001
NSCLC	63.8 (204/320)	25.0 [26]	<0.001
Breast Ca	25.0 (24/96)	56.6 [27]	0.012
Prostate Ca	23.9 (21/88)	52.2 [28]	0.022
All cancer	47.2 (359/760)	—	<0.001