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PROSPECTIVE USE OF AN OPTIMIZED RAS MUTATION DIAGNOSTIC IN AN ADJUVANT PHASE 2 TRIAL OF MUTATION SPECIFIC RAS-TARGETED IMMUNOTHERAPY (GI-4000) IN PANCREAS CANCER

Amber Britton, Sarah Oakes, Zhimin Guo, Yingnian Lu, Sue Speyer, Thomas King, Alex Franzusoff and David Apelian; GlobeImmune Inc., Louisville, Colorado

Introduction

The importance of *ras* genotyping has been highlighted in recent trials demonstrating poorer clinical outcomes following treatment with EGFR-targeted therapies in patients whose tumors harbor activating *ras* mutations. In non-small cell lung cancer (NSCLC), K-*ras* mutations are associated with primary resistance to tyrosine kinase inhibitor therapy (Pao. Can Chemo Pharm 2006). In another study patients with metastatic colorectal cancer with mutant K-*ras* who are treated with anti-EGFR antibody therapy have an ~33% worse survival than those treated with standard of care alone (Tol et al. NEJM 2009). In response to these findings ASCO released a provisional opinion on K-*ras* testing for colorectal cancer stating, "All patients with metastatic colorectal cancer…should have their tumor tested for K-*ras* mutations."

Prospective *ras* genotyping is likely to be similarly important in the screening of all cancers where *ras* mutations are found in a high percentage of tumors, including pancreas, colorectal, NSCLC, ovarian and other cancers. Inaccurate *ras* genotyping, including false negative results and failing to genotype for activating mutations in exons other than those encoding codons 12 and 13, may have significant consequences in clinical decision making.

GlobeImmune is developing GI-4000 Tarmogens® as a targeted immunotherapy designed to activate T cells to eliminate tumor cells expressing specific Ras mutations in exons 2 (G12V, C, D or R) or exons 3 (Q61L, R or H). *Ras* genotyping is a prerequisite for enrollment into the randomized double blind placebo controlled Phase 2 trial in newly resected pancreas cancer (GI-4000 plus gemcitabine vs. gemcitabine alone); see figure 1. Eligible patients are only treated with the specific GI-4000 product that matches the mutation identified in their tumor.

A variety of diagnostic methods have been developed to identify *ras* mutations in tumor biopsies, where PCR amplification and bi-directional sequencing of exon 2 of the *ras* gene is considered the 'gold standard' for genotyping. However, the gold standard diagnostic is susceptible to a risk of false negatives due to the presence of wild-type *ras* DNA from heterozygous tumor cells (which can interfere with the analysis of sequence chromatograms) and low overall tumor cell content in the paraffin embedded formalin fixed specimens. One method for the optimization of *ras* genotyping is the use of peptide-nucleic acid (PNA) oligomers to suppress PCR amplification of the wild-type *ras* sequences from patient biopsies. We have genotyped over 400 tumors from multiple cancers with and without PNA, and show that using PNA (P. Paulasova & F. Pellestor, Ann. Genetique 2004) oligomers and interrogating *ras* exon 3 sequences resulted in improvement in the qualitative and quantitative detection of *ras* mutations from patient biopsies.

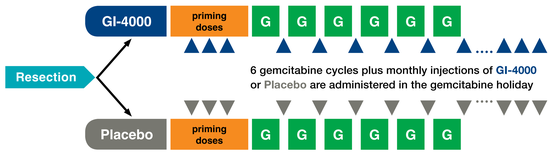
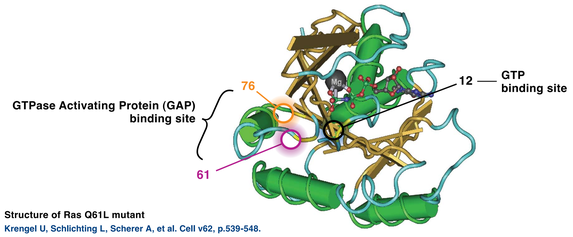


Figure 1: GI-4000-02 Phase 2 study design

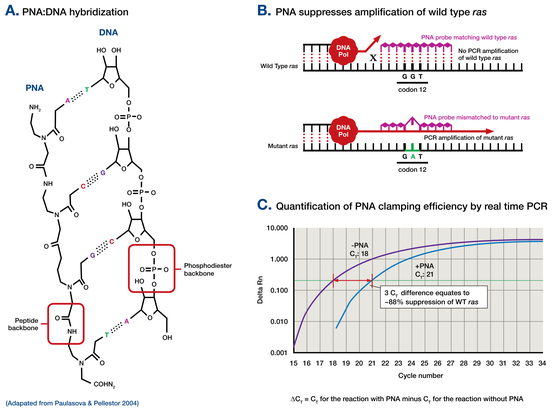
GI-4000-02 is an ongoing, double-blind trial evaluating GI-4000 vs. placebo in combination with 6 cycles of adjuvant gemcitabine in patients with successfully resected pancreas cancer (R0 or R1). Subjects are prospectively tested to identify the specific *ras* mutation in their tumor. This study will enroll at least 100 patients at 40 US centers and 15 international centers. Subjects receive 3 priming doses of study drug (matching their mutation) or placebo prior to initiation of gemcitabine therapy, followed by monthly doses of study drug or placebo, continuing until disease recurrence. Recurrence-free survival (RFS) is the primary endpoint, and overall survival (OS) is a key secondary endpoint.



Structure of Ras Q61L mutant
Krengel U, Schlichting L, Scherer A, et al. Cell v62, p.539-548.

Figure 2: Ras as a cancer target

- *ras* is most commonly mutated oncogene in human cancer (>160,000 cases/year in the U.S.)
  - Pancreas, colorectal, NSCLC, ovarian
- "Hotspot" mutations activate cellular proliferation
- Mutations predict poor prognosis with EGFR-targeted drugs & chemotherapy
- GI-4000 Tarmogen products target common mutations in codons 12 and 61



(Adapted from Paulasova & Pellestor 2004)

$\Delta C_T = C_T$ for the reaction with PNA minus C_T for the reaction without PNA

Figure 3: What is PNA clamping?

(A) PNA forms duplexes with DNA that are more stable than double-stranded DNA because the PNA backbone is more flexible and lacks negative charges that contribute to inter-strand repulsion; (B) single base mismatches in PNA:DNA hybrids cause large Tm decreases enabling discrimination between wild type and mutant *ras* alleles; (C) real-time PCR analysis of PNA clamping efficiency. With a ΔC_T of 3, as shown in the figure, the blocking efficiency of wild type *ras* amplification would be ~88%. [% block = $(1\text{-}2^{-\Delta C_T})$]

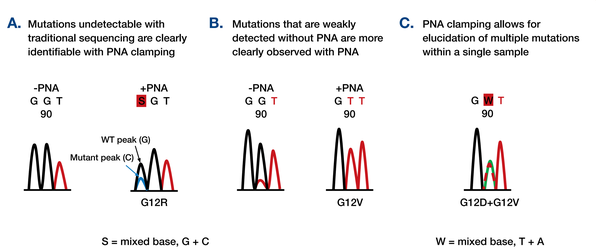


Figure 4: PNA clamping improves detection of K-*ras* mutations

PNA clamping is definitive at improving sensitivity of K-*ras* mutation identification and the detection of multiple mutations in a single tumor. These traces were taken from electrophoretograms of patient biopsy genotyping results.

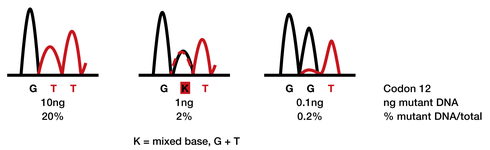


Figure 5: PNA clamping improves detection sensitivity

The sensitivity of the PNA clamping method was evaluated by mixing different amounts of genomic DNA from the cell line SW480 (homozygous for a G12V mutation in K-*ras*) into human genomic DNA that contains only wild type K-*ras*. These experiments determined that the PNA clamping method is capable of detecting mutations from as little as 1ng of mutant K-*ras* DNA, which if analyzed in a patient biopsy sample, corresponds to the successful detection of the mutation even if only 4% of cells in the biopsy were tumor cells encoding a heterozygous *ras* mutation.

	Patient number									
	1	2	3	4	5	6	7	8	9	10
Result with PNA	G12S	G12V	G12R	G12V	G12D	G12D	G12V	G12R	G12D	G12V
Result without PNA	WT	G12V	WT	G12V	G12D	WT	G12V	G12R	WT	G12V

Table 1: PNA clamping reduces false negative rate

A concordance study of 10 clinical tumor samples using bidirectional sequencing with and without PNA clamping showed a 40% false negative rate for mutations in *ras* when PNA clamping was not employed.

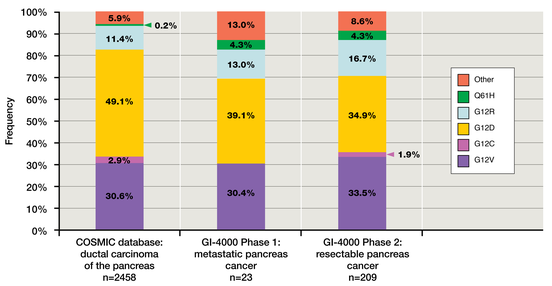


Figure 6: Frequency of K-*ras* mutations: expected vs. observed

Data obtained from the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) show the most frequent mutations in pancreas cancer to be G12D, G12V, and G12R. COSMIC likely under-reports exon 3 mutations because the majority of patients were not screened at this locus.

Tumor source	G12V	G12C	G12D	G12R	Q61H	Other	Mutant samples	Total samples	% mutant
COSMIC database	753	71	1206	280	4	144	2458	3458	71%
Phase 1 trial subjects (analyzed without PNA)	7	0	9	3	1	3	23	32	72%
Phase 2 trial subjects (analyzed with PNA)	70	4	73	35	9	18	209	242	86%

Table 2: Sequencing results in biopsies from pancreas cancer patients from the GI-4000 program

The genotyping method was successful for all 274 samples tested in Phase 1 and Phase 2 trials. An increase in the percentage of mutant *ras* samples was observed after introduction of PNA clamping.

Conclusions

1. Accurate *ras* genotyping is essential for patient enrollment in trials of GI-4000 Tarmogen immunotherapy targeting tumors with specific *ras* mutations
2. False negative reporting of *ras* mutations and the decision to treat with EGFR-targeted therapies plus chemotherapy could either provide no patient benefit or result in worse patient prognosis
3. PNA-mediated suppression of wild type *ras* amplification improved qualitative and quantitative detection of *ras* mutations from pancreas cancer biopsies
4. The use of PNA clamping plus bi-directional sequencing maintains the ability to detect multiple mutations and less common *ras* mutations
5. The 4% incidence of Q61H *ras* mutations in pancreas cancer is much higher than previously reported
6. Inclusion of PNA and interrogation of *ras* exons 2 and 3 reduces the probability of false negative reporting from *ras* genotyping by at least 14% in this study

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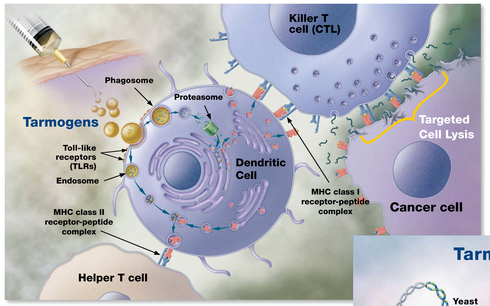
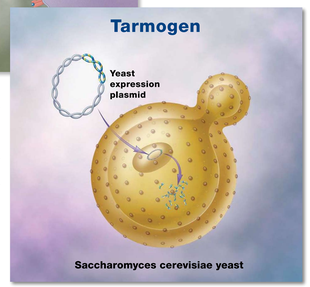
Active immunotherapy with yeast-based Tarmogens

Tarmogens are whole, heat-killed recombinant *Saccharomyces cerevisiae* yeast modified to express one or more protein targets that stimulate the immune system against diseased cells. The target antigens are markers of diseased cells and can be conserved viral proteins, mutated proteins unique to cancer cells, or proteins over-expressed in cancer. To create a new Tarmogen, DNA encoding target protein antigens is engineered into a yeast expression plasmid. The heat-inactivated yeast, with the target protein inside, is administered as the Tarmogen product. Tarmogens stimulate the innate and antigen-specific immune system to produce a highly specific and potent T cell response against the diseased cell, with little or no impact on healthy cells.[1]





Tarmogens are administered subcutaneously and are avidly taken up by antigen presenting cells (APCs), such as dendritic cells and macrophages in a process mediated by Toll-like receptors (TLRs) found on the cell surface. Uptake of Tarmogens activates the APCs and results in their migration to lymph nodes and their production of immune-stimulating cytokines.[2,3]

Tarmogens are degraded inside APCs within hours and the target antigens are presented by MHC class I and II receptors on the APC surface. Tarmogens are initially digested in phagosomes, whereupon the antigens are delivered to the cytosol, and these proteins are cleaved by proteasomes into small peptides. These small peptides are loaded into newly folded MHC class I receptors in the secretory pathway of the APC. The peptide-MHC I receptor complex is shuttled to the surface of the APC, where the antigenic peptides are presented to CD8+ killer T cells (causing activation of these cells). Tarmogens are also digested in endosomes, and the product-associated peptides are loaded into MHC class II receptors for antigen presentation to CD4+ helper T cells (causing activation of these cells).[2,3]

Therapeutic benefit from the Tarmogen is driven by the targeted activation of the immune system. Tarmogens activate killer T cells capable of locating and destroying the target cancer or virally-infected cells. Repeated dosing with Tarmogens further increases the number of T cells available to eliminate diseased cells. In summary, Tarmogens couple the innate immune response to yeast with potent activation of antigen-specific cellular immune responses against cancer cells or virally infected cells.[3-4]

[1] Munson et al. "Coupling Innate and Adaptive Immunity with Yeast-Based Cancer Immunotherapy" Chapter 9; Cancer Vaccines and Tumor Immunity. January 2008
[2] Bernstein et al. "Recombinant Saccharomyces cerevisiae (yeast-CEA) as a potent activator of murine dendritic cells." Vaccine (2008) 26, 509-521.
[3] Remando et al. "Human Dendritic Cell Maturation and Activation by a Heat-Killed Recombinant Yeast Vector Encoding Carcinoembryonic Antigen." Vaccine (2009) 27, 987-994.
[4] Wansley et al. "Vaccination with a Recombinant Saccharomyces cerevisiae Expressing a Tumor Antigen Breaks Immune Tolerance and Elicits Therapeutic Antitumor Responses" Clinical Cancer Research. Clin Can Res (2008) 14,4316-4325.
[5] Haller et al. "Whole recombinant yeast-based immunotherapy induces potent T cell responses targeting HCV NS3 and Core proteins" Vaccine (2007) 25, 1452-1463.

Abstract

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Amber Britton, Sarah Oakes, Zhimin Guo, Yingnian Lu, Sue Speyer, Thomas King, Alex Franzusoff and David Apelian

GlobeImmune Inc., Louisville, CO

Background: Ras mutations are important predictive markers for EGFR-targeted treatment of non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) and are found in 71% of pancreas ductal adenocarcinomas (COSMIC database). GI-4000 is a targeted immunotherapy designed to eliminate tumor cells expressing specific Ras mutations in exons 2 (G12V, C, D or R) or exons 3 (Q61L, R or H).

Ras genotyping is a prerequisite for enrollment into the randomized double blind placebo controlled Phase 2 trial in newly resected pancreas cancer (GI-4000 plus gemcitabine vs. gemcitabine alone). Eligible patients are only treated with the GI-4000 product that matches the mutation identified in their tumor. The gold standard diagnostic method, namely PCR amplification and double strand sequencing limited to *ras* exon 2 is susceptible to a risk of false negatives due to the presence of wild-type ras DNA from heterozygous tumor cells and low tumor cell content in the paraffin embedded formalin fixed specimens. Eliminating false negatives should improve clinical decision making for all mutant *ras* driven cancers. Here we describe how use of an optimized *ras* genotyping method improved patient screening.

Experimental procedures: Tissue was obtained by surgical resection of tumors from 274 subjects with Stage I and II pancreas cancer to date from sites in US, India and Bulgaria. Tumors were genotyped for K-*ras* exons 2 and 3 using an optimized bi-directional sequencing of DNA from nested PCR amplification with peptide-nucleic acid (PNA) oligomer clamping.

Results: Compared to the gold standard *ras* genotyping method, the inclusion of PNA oligomers suppressed amplification of wild type *ras* sequences (as previously reported) and substantially reduced the false negative rate. This optimized method detected *ras* mutations down to 1ng of genomic DNA, and to 2% heterozygous mutant tumor cells in the sample. Using the optimized method, tumor genotyping revealed that 86% of pancreas cancers screened for the Phase 2 study had K-*ras* mutations, predominantly G12V or G12D. In addition, tumors harbored G12R (13%) or Q61H (4%) mutations at greater frequencies than previously reported. Non-canonical mutations were also detected, highlighting a potential source of false negative results for assays with restricted mutation coverage.

Conclusions: Prospective *ras* testing in an ongoing Phase 2 study of newly resected pancreas cancer demonstrates the importance of optimizing the diagnostics used to screen tumors for the purpose of personalized cancer therapies. We observed substantial reduction in the rate of false negatives and increased detection of non-canonical mutations in ras. Improved sensitivity and specificity of *ras* diagnostic methods are also predicted to have important implications in treatment decisions and outcomes in the context of EGFR-targeted approaches for NSCLC and CRC.