TRANSNATIONAL PATTERNS OF PANCREAS CANCER RAS MUTATIONS AND DISCOVERY OF A NEW RAS MUTATION WITH ONCOGENIC SYNERGY WHEN FOUND WITH RAS CODON 12 MUTATIONS

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Introduction

Activating mutations in the Ras oncogene family occur in 30% of all human cancers, with higher incidences in adenocarcinomas of pancreas, colorectal cancer, and non-small cell lung cancer. Mutations at Ras residues 12, 13, or 61 are known to drive uncontrolled cell proliferation and tumorigenesis.

GlobeImmune is developing cancer immunotherapy products designed to activate T cells capable of killing tumors expressing mutant Ras. The GI-4000 product series consists of four different yeast Tarmogens®, each targeting mutated Ras antigens with a codon 61 mutation (Q61L, Q61R or Q61H) plus one of four codon 12 mutations (G12V, G12C, G12D, or G12R). A Phase 1 clinical trial in late stage (III/IV) cancer patients showed that GI-4000 was well-tolerated and elicited Ras Mutation-specific immune responses. An international, placebo-controlled Phase 2 trial evaluating GI-4000 in newly resected (early stage) pancreas cancer with adjuvant gemcitabine is underway. Enrollment in these GI-4000 trials requires tumor genotyping to identify the patients' Ras mutation for administration of the relevant GI-4000 product to match the tumor-associated Ras mutation.

The international genotyping analysis to support the GI-4000 clinical trials provides valuable information on the frequency and spectrum of Ras mutations as a function of geographic location. Ras genotyping results for GlobeImune's GI-4000 clinical trials, which were generated from over 200 subjects in Bulgaria, India, Taiwan, and the US, are reported here. Because direct sequencing of *ras* DNA was used for this analysis, including the hot spot mutational regions around codons 12, 13 (exon 2), and 61 (exon 3), the incidence of each hot-spot alteration as well as non-canonical mutations was evaluated. This led to the discovery of a novel mutation at codon 76, whose tumorigenic role alone or in combination with hot spot G12 mutations was investigated in pre-clinical models and the findings are discussed in relation to the human genotyping results.

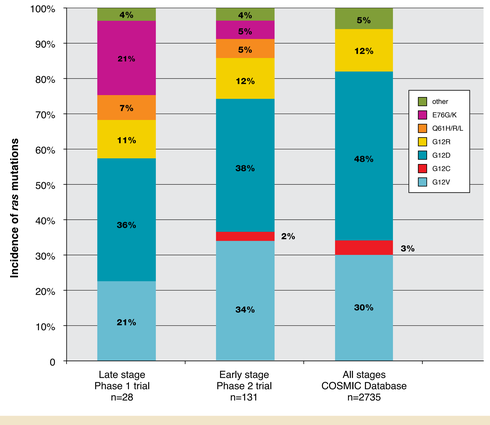


Fig. 1. New patterns of K-*ras* mutations in pancreas cancer

K-ras genotyping results from pancreas tumor samples from the GlobeImmune Phase 1 (late-stage metastatic disease) and Phase 2 (newly resected early-stage cancer) clinical trials of GI-4000 are compared to mutation frequencies obtained from the COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic). Similar *ras* mutation frequencies are evident in the three data sets, except for the unexpected prevalence of codon 61 mutated *ras* and the occurrence of a newly identified mutation at codon 76.

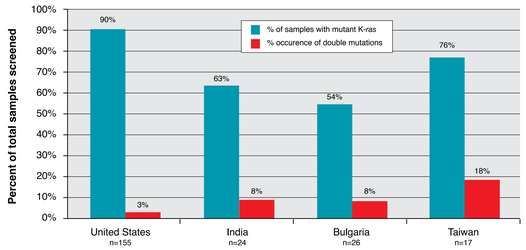


Fig. 2. Incidence of *K-ras* mutations varies between countries

K-ras mutations in pancreas tumors occur at different frequencies across countries (blue bars) The incidence of double mutations in K-*ras* (red bars) also varies for each country. These double mutations consisted of predominantly G12 + E76 mutations in the US; double G12 mutations were detected in samples from India and Bulgaria, whereas double mutations from Taiwan samples consisted of a mutation at G12 and an alteration at G13 or non-canonical residues.

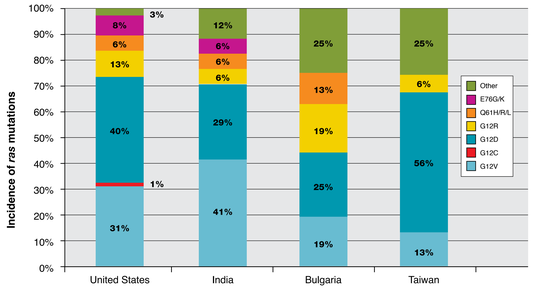
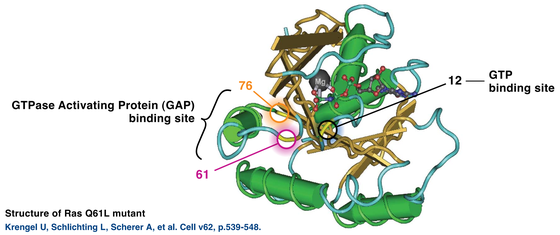


Fig. 3. The spectrum of *K-ras* mutations differs by country.

The relative frequency of mutant *K-ras* genotypes (V,C,D, and R) at codon 12 differs between countries. Mutations at codon 76 were found only in patients from the US and India. Mutations at codon 61 are more frequent than previously reported in pancreas cancer (COSMIC database), especially in samples from Bulgaria, but were absent in the Taiwan samples. Bulgarian patients also showed a higher incidence of non-canonical mutations. Previous studies reported a predominant G12V genotype in pancreas cancer patients in Taiwan (Wang JY 2002, *Cancer Letters*), while the G12D mutation has been documented as predominant in pancreas cancer patients from mainland China (Wei S 2005, *J of Gastro Hepato.* and Dong M 2000, *Cancer Epidem, Biomarkers & Prevention*). In this study however, tissue from Taiwanese patients showed G12D predominance. It was verified that the Taiwan samples were indeed obtained from patients of mainland Chinese descent.

Fig. 4. Residues 12, 61 and 76 affect domains of Ras function.



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

Clone (K-*ras* genotype transfected into BALB/c-3T3 cells)	mRNA transcript of transfected *ras* gene (by RT-PCR)	Exogenous Ras protein expression (western blot)	Colony formation in soft agar
wild-type (WT) K-*ras*	+	+	-
G12V	+	+	++++
G12V + E76G	+	+	+++++
E76G	+	+	-
E76K	+	+	++
pUP (empty vector)	-	-	-
untransfected	-	-	-

Table 1. Ras mutations cause transforming phenotypes *in vitro*

Single or double *ras* mutations were introduced into the mouse *K-ras* gene by site-directed mutagenesis and then transfected into BALB/c-3T3 fibroblasts.

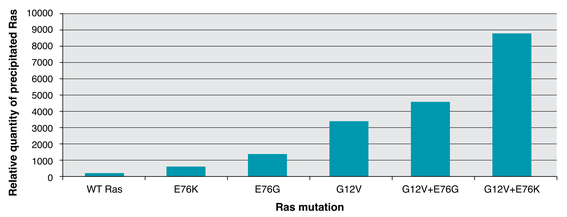


Fig. 5. Ras mutations at residues G12 and E76 have synergistic effects on GTP binding

Total protein extracted from BALB/c-3T3 cells (expressing wild-type or mutant Ras proteins) was incubated with Raf-glutathione-s-transferase-coupled agarose beads. The Ras protein was eluted from the beads and the amount of precipitated Ras was quantified by western immunoblot.

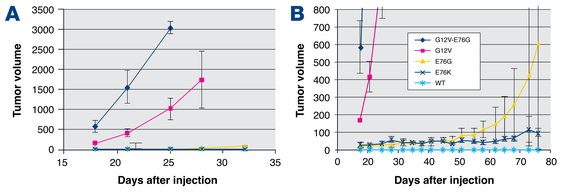


Fig. 6. Tumorigenicity assay with BALB/c-3T3 nude mice

Five million cells were injected s.c. into 4-6 week-old BALB/c nude mice and tumor growth was measured. Panel A reveals oncogenic synergy in the growth of tumors carrying Ras mutations at both codon 12 and codon 76. Panel B is the expanded detail of panel A, showing the growth of individual E76 mutant-transfected clones relative to BALB/c-3T3 cells transfected with wild-type *ras*.

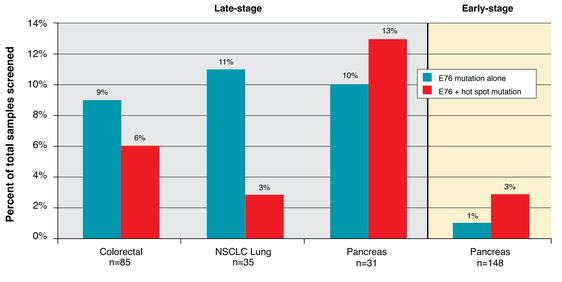


Fig. 7. Mutations in *ras* codon E76 are more frequently observed in late-stage disease

The E76 mutation was frequently detected along with a hot spot G12 mutation in *K-*, *N-*, and *H-ras*. Based on the preclinical results shown above, these double mutations may act synergistically to drive cancer progression.

Conclusions

1. The incidence and distribution of *K-ras* mutations in pancreas cancer differs in patients from different countries, underscoring the essential role for direct genotyping in pancreas cancer treatment paradigms.
2. A new Ras E76 mutation was identified and found to be more prevalent in late-stage cancers.
3. Ras E76 mutations synergize with Ras G12 mutations to exacerbate the aggressive tumor growth phenotype in preclinical models.
4. Ras E76 mutations represent a new cancer biomarker and target for cancer immunotherapy.

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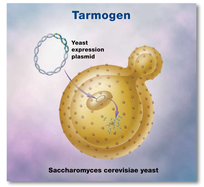
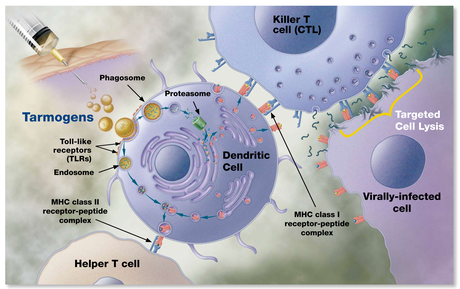
Cancer immunotherapy with yeast-based Tarmogens

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 final 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]

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]

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]

For more information, visit www.globeimmune.com.

[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] Wansley et al. "Vaccination with a Recombinant Saccharomyces cerevisiae Expressing a Tumor Antigen Breaks Immune Tolerance and Elicits Therapeutic Antitumor Responses" Clinical Cancer Research. July 2008.
[4] 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

TRANSNATIONAL PATTERNS OF PANCREAS CANCER RAS MUTATIONS AND DISCOVERY OF A NEW RAS MUTATION WITH ONCOGENIC SYNERGY WHEN FOUND WITH *RAS* CODON 12 MUTATIONS

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Background: Patterns of K-Ras oncoprotein mutations at amino acids 12, 13, or 61 drive uncontrolled cell proliferation and tumorigenesis. We have developed yeast-based immunotherapy targeting Ras point mutations (GI-4000 tarmogens) and we analyzed the incidence of specific *ras* point mutations among patients with pancreas cancer in Bulgaria, India, Taiwan and the US.

Experimental procedures: Tumor biopsies were obtained from 222 patients in Bulgaria, India, Taiwan and the US. DNA sequences were characterized for tumor-associated mutations in *K-ras* exons 2 and 3 by nested PCR amplification including peptide-nucleic acid oligomer clamping, followed by DNA sequencing. Single G12V, Q61R, E76G or E76K mutations or double G12V+E76G, Q61R+E76G mutations were encoded into the mouse K-*ras* gene then transfected into BALB/c-3T3 fibroblast cells. The transfected cells were seeded for growth in soft agar or implanted into BALBc athymic nude mice for tests of tumorigenesis. The intracellular content of GTP-bound Ras protein, a marker of Ras activation, was assayed with cells transfected to express extra copies of wild type Ras, or Ras with single or double mutations.

Results: Notable differences from previously reported patterns included i) a different profile of *ras* point mutation frequencies found in pancreas cancer in different countries; ii) the unanticipated frequency of amino acid 61 Ras mutations (Ras Q61H); and iii) the discovery of a new Ras mutation at amino acid 76 (Ras E76G or E76K) that was detected in patient tumors as a single mutation or simultaneously with amino acid 12 *Ras* mutations.

The role of Ras oncoprotein harboring E76 mutations alone or in combination with G12 mutations in tumorigenesis was investigated in preclinical studies. Cells transfected with *K-ras* gene encoding single G12V, Q61R or E76K mutations or double G12V+E76G, Q61R+E76G or Q61R+E76K mutations all exhibited the transformed phenotype by forming colonies in soft agar. Cells expressing singly mutated Ras had increased intracellular levels of GTP-bound Ras protein compared to overexpression of wild type Ras protein and GTP-bound Ras levels were further amplified in cells expressing Ras with double G12+E76 mutations. To confirm tumorigenesis in vivo, tumors stemming from double G12V+E76G mutations implanted into BALBc mice showed more aggressive growth rates compared to those expressing any single mutant Ras.

Conclusions: Tumor genotyping reveals differences in transnational incidence of pancreas cancer-associated *ras* mutations and led to discovery of Ras-E76 mutations. Tumors expressing double Ras G12+E76 mutations exhibit more aggressive growth in preclinical studies and identifying *ras* double mutations may be prognostic of clinically more aggressive malignancies. These results highlight the importance of *ras* genotyping in all countries and predict that Ras E76 mutations represent a new cancer biomarker and target for cancer immunotherapy.