Immune Gene Expression, Bayesian Network and Genetic Mutation Analysis in Advanced NSCLC Treated with Immunotherapy

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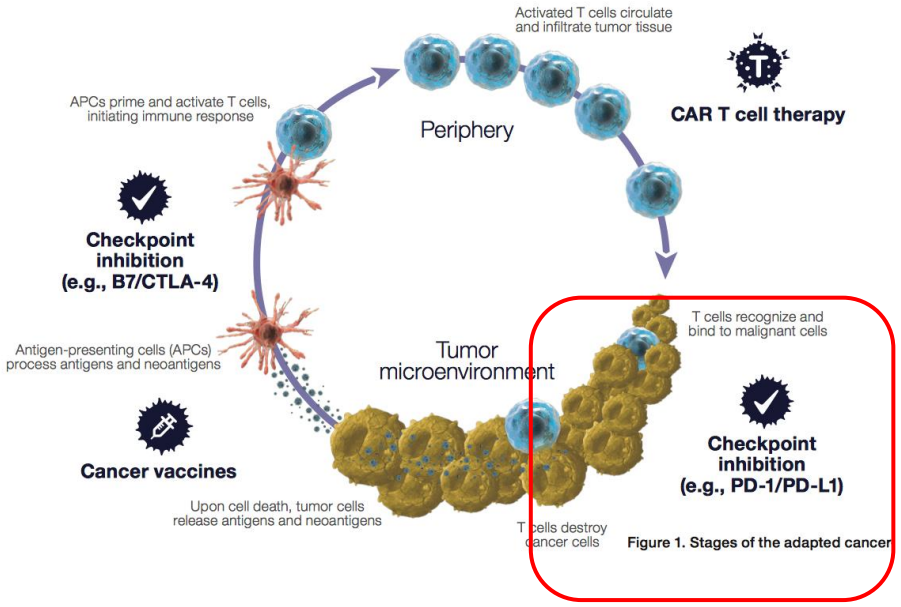
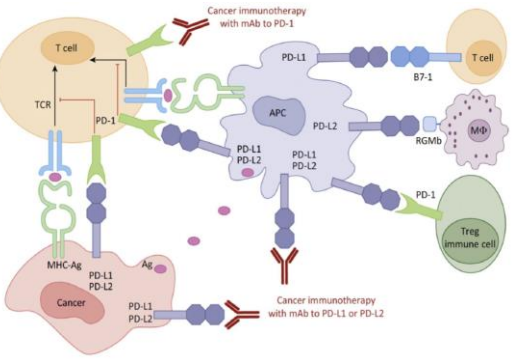
- Speaker was provided travel and hotel support by Thermo Fisher Scientific for this presentation, but no remuneration

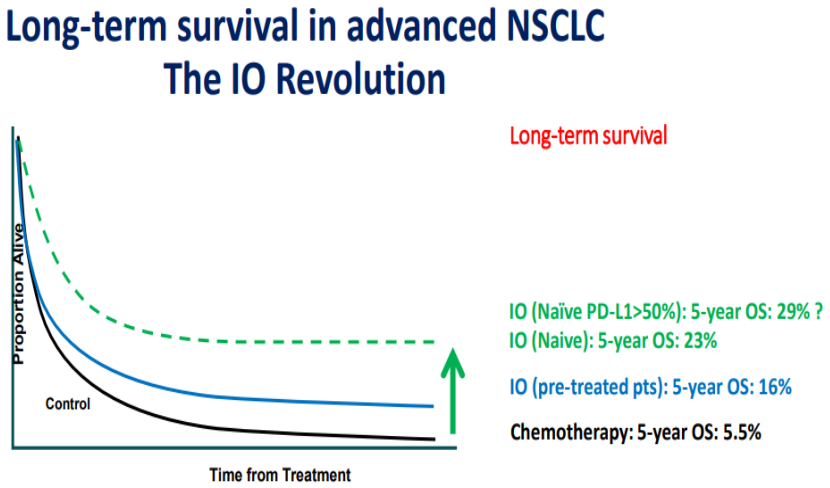
Background and Rationale

- Immune checkpoint inhibitors (ICIs) have revoluzionized the therapeutic paradigm for different types of cancer including NSCLC
- Unfortunately, durable benefit is limited to a minority of patients
- The only adopted predictive biomarker, PD-L1 IHC testing, suffers from some limitations
- Better understanding of biomarkers associated with response to ICIs is needed



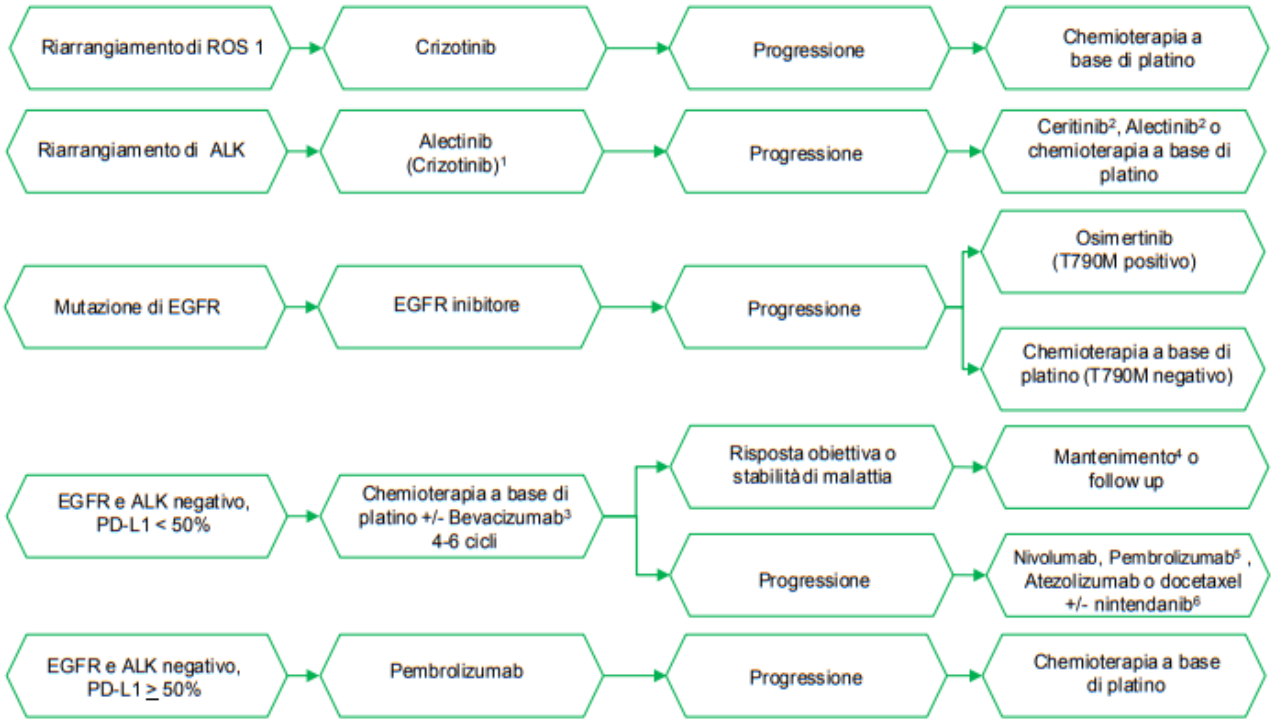


Kim C. Ohaegbulamet al. Trends in Molecular Medicine 2015, Vol. 21, No. 1



LINEE GUIDA 2018

NSCLC: malattia metastatica



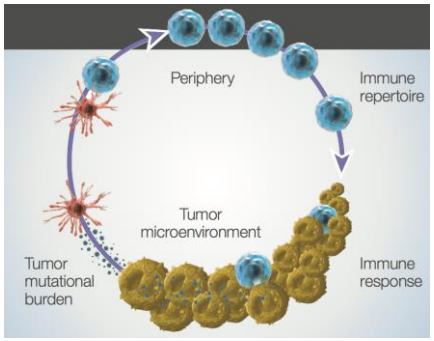
1. Crizotinib è risultato inferiore ad Alectinib in un confronto randomizzato diretto
2. In pazienti in progressione a Crizotinib
3. Solo nell'istologia non squamosa; il trattamento con Bevacizumab può essere mantenuto fino a progressione
4. Mantenimento con Pemetredex, solo nell'istologia non squamosa
5. Solo se PD-L1 ≥ 1%
6. Solo nei pazienti con istologia adenocarcinomatosa, soprattutto in caso di progressione di malattia entro 9 mesi dall'inizio della terapia di I linea.

NEAR FUTURE

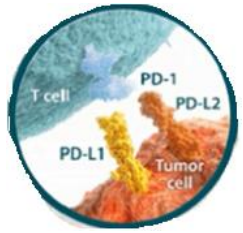
Controversy in advanced NSCLC: «the best first»

- First-line treatment in wild-type advanced NSCLC: CT vs CT+I/O or I/O vs CT+I/O (in selected patients)

Investigating I-O Biomarkers: inflamed tumors



- Programmed death ligand 1 (PD-L1)
- Tumor-infiltrating lymphocites (TILs)
- Expression gene signature
- Tumour Mutational Burden



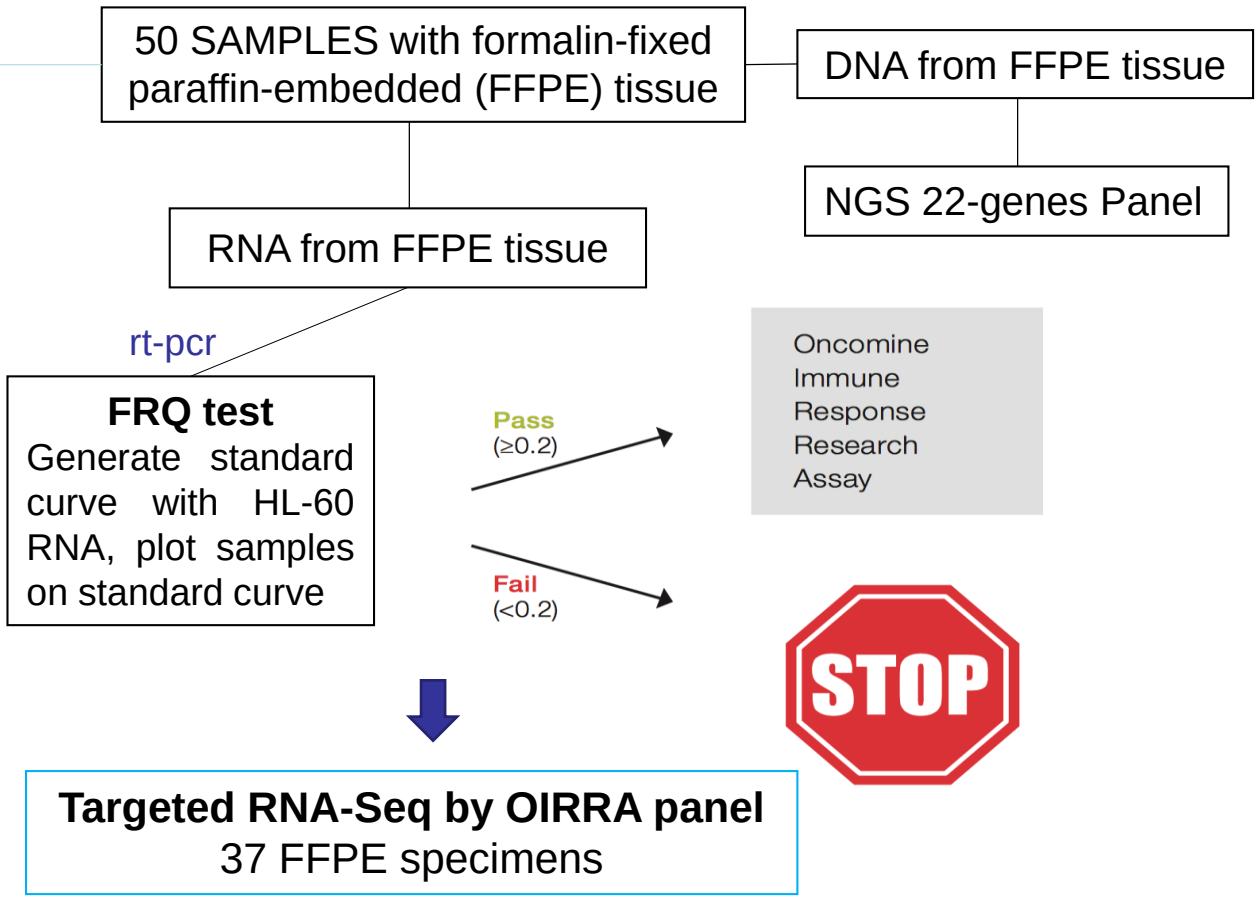
they may correlate with clinical response

- Early reports sugges PD-L1 immunohistochemistry, T-cell infiltration levels, high tumor mutational burden (TMB) and gene expression profiling (GEP) may correlate with clinical response
- Emerging biomarkers such as TMB and GEP could be predictive of clinical benefit across diverse human cancers
- There is a compelling need for a better understanding of factors that would predict response and progression

IO BIOMARKERS (BEYOND PD-L1) NEEDED

Study Disegn

Stage IV NSCLC samples treated with Nivolumab 3 mg/kg every 2 weeks; >1 prior line of anticancer therapy for advanced disease; ongene addicted samples included; <u>tumour tissue available for biomarker testing</u>



<u>Study Objectives</u>

- ✓ To assess immune gene-expression (GEX) and genetic mutation profiles
- ✓ To evaluate association of GEX with clinical response to immunotherapy in advanced NSCLC samples treated with ICI

Material and Method

Sample collection

FFPE tissue samples from advanced NSCLCs, treated with nivolumab starting from the 2° line of therapy

Tab. 1 Clinical-pathological characteristics of patients

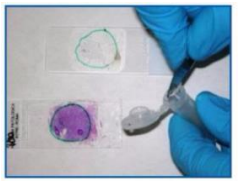
CHARACTERISTICS	Samples N=37	%
Median Age, Years (range)	64 (46-82)	
Sex		
Female	11	29,7
Male	26	70,3
Performance Status*		
0	10	27,0
1	23	62,0
2	4	1,0
Smoking History		
Never smoker	6	16,2
Current Smoker	31	83,8
Histology		
Adenocarcinoma	27	73,0
Squamous-cell carcinoma	10	27,0
Stage		
IV	37	100
Genetic alterations**		
WT	13	48,1
EGFR mut	5	18,6
KRAS mut	9	33,3
Type of immune checkpoint inhibitor (ICI)		
Nivolumab	37	100
Clinical Response to nivolumab***		
Responder (R)	15	40,0
No-Responder (NR)	22	60,0
N. lines of therapy before nivolumab		
1	22	59,5
2	11	29,7
3	3	8,1
4	1	2,7

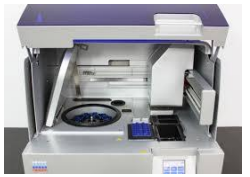
* Before nivolumab
** Adenocarcinoma histology
*** Responder: CR/PR/ SD≥6 months; No-Responder: PD, SD<6 months
Assessed by Recist 1.1

RNA/DNA isolated using
- **RNeasy FFPE KIT**
- **QIAamp DNA FFPE tissue Kit**

automatically purified by QIAcube robotic workstation

1) Tumor sample





- RNA and DNA extracted from FFPE sample
- QIA**c**ube robotic workstation

37 FFPE tissue samples

Targeted RNA-Seq by Oncomine™ Immuno Response Assay (OIRRA)

Gene network analysis

Cancer somatic mutation

Material and Method

Oncomine™ Immuno Response Assay (OIRRA)

RNA samples were performed by RNA-Seq using the OIRRA (ThermoFisher Scientific) on **Ion Torrent PGM** and **Transcriptome Analysis Console (TAC) v4.0 Software**. The panel measures the expression level of 395 genes.

2) Library and template preparation

- Ion Chef™ system
- AmpliSeq™ RNA panel for immune Response with low imput 10ng RNA

3) Next-generation sequencing

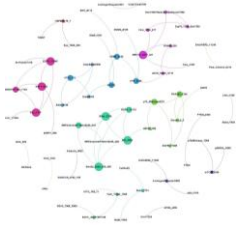
Ion PGM System Sequencer

4) Analyze Data

- Ion Reporter Software
- Torrent Suite™
- Transcriptome Analysis Console (TAC) 4.0 Software

Gene network analysis

Gene network analysis based on Bayesian algorithm was performed by **GeneMANIA database** querying with the genes selected through mRNA expression analysis



Cancer somatic mutation

DNA samples were assessed by **Ion Ampliseq Colon and Lung Cancer Research Panel v.2** that detected cancer somatic mutation of 22 genes on PGM Instrument starting from 10 ng of DNA

For research use only. Not for use in diagnostic procedures

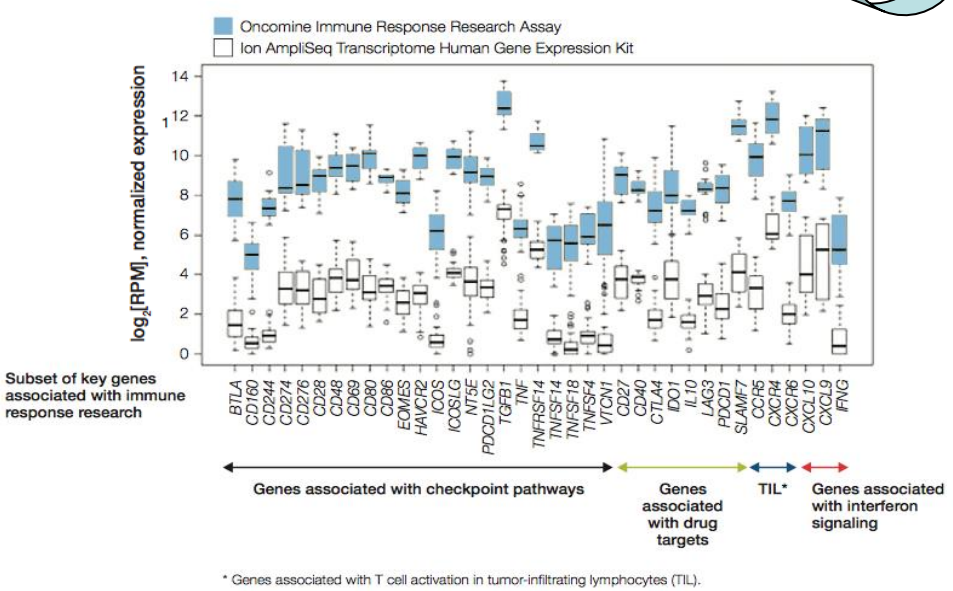
OIRRA panel

Oncomine™ Immune Response Research Assay

Analysis of the immune pathway and low-expressing genes from samples with 10 ng **FFPE RNA**

OIRRA content includes **395 genes** across **36 functional annotation groups**

Groups are comprised of genes associated with **lymphocyte regulation - cytokine signaling - lymphocyte markers checkpoint pathways and tumor characterization**

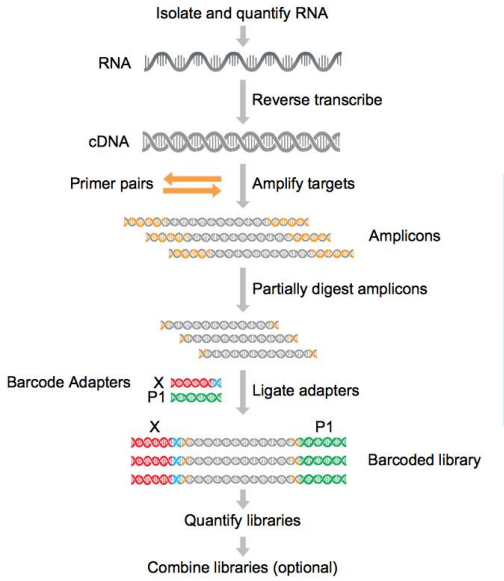


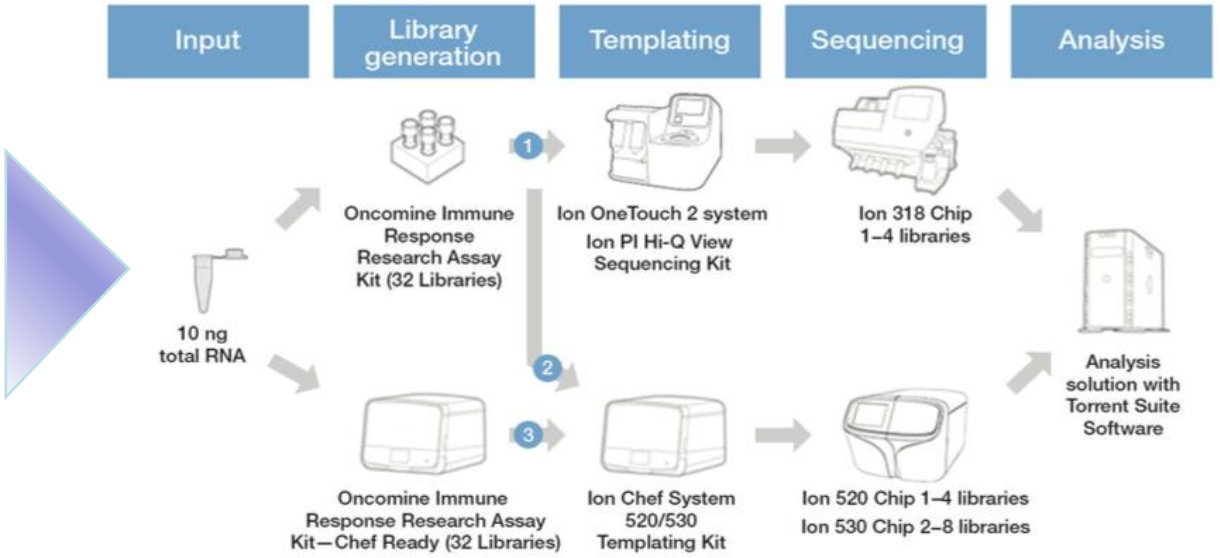
* Genes associated with T cell activation in tumor-infiltrating lymphocytes (TIL).

Function	Number of genes
Antigen presentation	3
Antigen processing	19
Innate immune response	11
Leukocyte inhibition	2
Leukocyte migration	5
Lymphocyte activation	2
Lymphocyte development	3
Lymphocyte infiltration	46
B cell receptor signaling	3
T cell receptor signaling	6
T cell regulation	9
TCR coexpression	19
Chemokine signaling	10
Cytokine signaling	15
Interferon signaling	8
Type I interferon signaling	8
Type II interferon signaling	23
Housekeeping	11

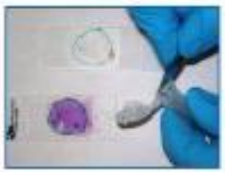
Function	Number of genes
B cell marker	11
Dendritic cell	7
Dendritic cell, macrophage	6
Helper T cells	8
Macrophage	5
Myeloid marker	7
Neutrophil	5
NK cell activation	8
NK cell marker	4
T cell differentiation	2
Checkpoint pathway	30
PD-1 signaling	9
Drug target	21
Adhesion, migration	14
Apoptosis	4
Proliferation	10
Tumor antigen	17
Tumor marker	27

OIRRA NGS Workflow





1) Tumor sample



- QIAcube robotic workstation
- RNA and DNA extracted from FFPE sample

2) Library and template preparation

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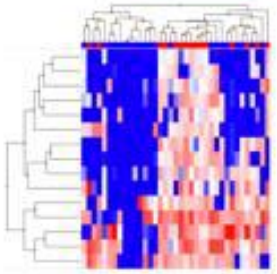
3) Next-generation sequencing

Ion PGM System Sequencer

4) Analyze Data

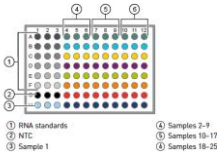
- Ion Reporter Software
- Torrent Suite™
- Transcriptome Analysis Console (TAC) 4.0 Software

5) NGS Gene expression



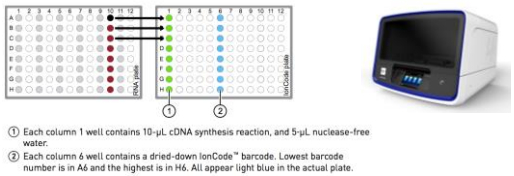
OIRRA Workflow

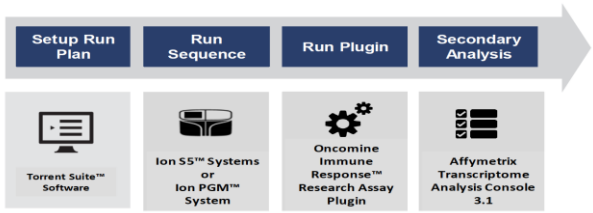
- **Real-time PCR** for RNA quality assessment
  RNA **from FFPE Tissue → TEST FRQ**



37 samples FRQ≥0.2 (74%)
13 samples FRQ<0.2 (26%)

- **Reverse transcription** and target amplification
  10 ng of RNA →
  OIRRA Chef-Ready Kit

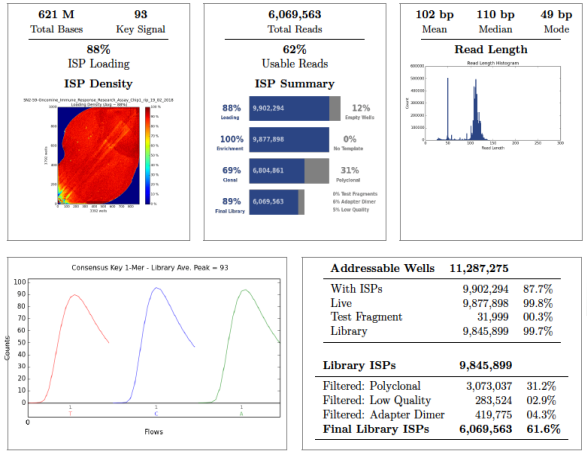




Primary Analysis
Torrent Suite Analysis

Run Report for
Auto_user_SN2-59-Oncomine_Immune_Response_Research_Assay_Chip1_rip_19_02_2018_172

Run Summary



Addressable Wells	11,287,275	
With ISPs	9,902,294	87.7%
Live	9,877,898	99.8%
Test Fragment	31,999	00.3%
Library	9,845,899	99.7%
Library ISPs	**9,845,899**	
Filtered: Polyclonal	3,073,037	31.2%
Filtered: Low Quality	283,524	02.9%
Filtered: Adapter Dimer	419,775	04.3%
Final Library ISPs	**6,069,563**	**61.6%**

CHP files
normalized by RPM
(Reads per Milion)

Barcode Name	Sample	Bases	≥ Q20	Reads	Mean Read Length	Read Length Histogram
No barcode	none	17,322,592	16,413,061	157,675	109 bp	
IonCode_0101	IMM-1-R	12,380,904	12,026,628	145,870	84 bp	
IonCode_0102	IMM-14-R	121,067,412	117,846,709	1,207,478	100 bp	
IonCode_0103	IMM-16-R	92,606,638	90,254,006	891,754	103 bp	
IonCode_0104	IMM-19-R	83,100,978	80,829,017	840,868	98 bp	
IonCode_0105	IMM-20-R	139,922,211	136,613,247	1,283,144	109 bp	
IonCode_0106	IMM-23-R	81,534,226	79,081,112	860,448	94 bp	
IonCode_0107	IMM-24-R	10,755,172	10,476,586	106,880	100 bp	

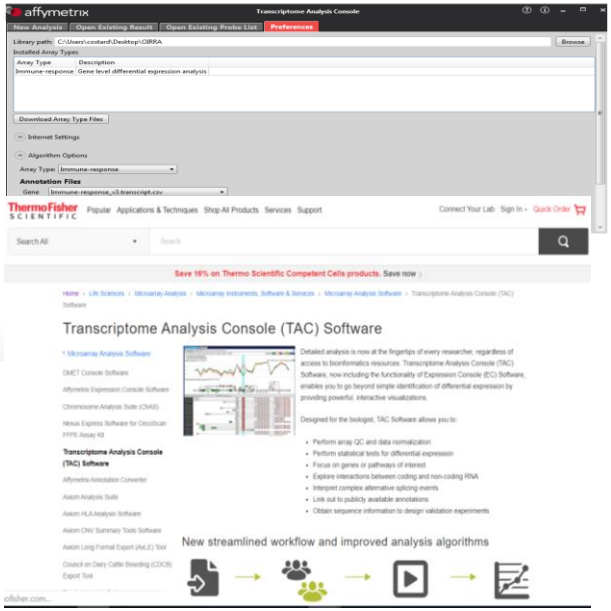
1

iontorrent
by Thermo Fisher Scientific

Ion Torrent PGM
2 Chip318 x8 samples

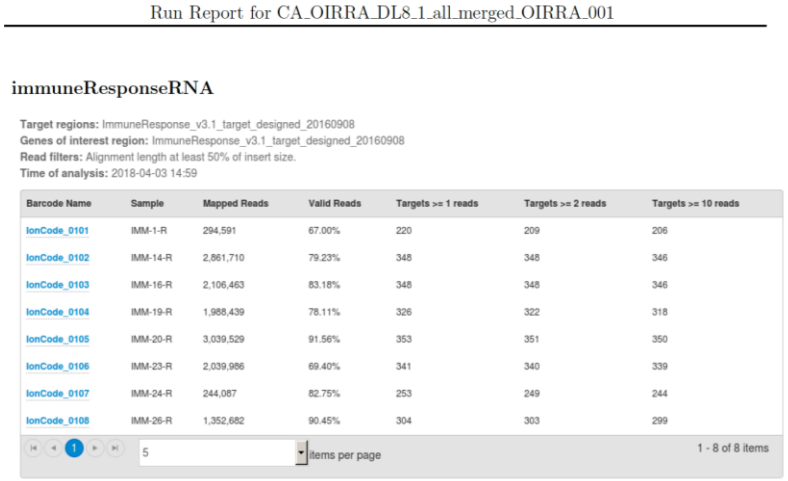
Secondary Analysis
TAC analysis

- Open TAC
- Go to **Preferences**
- Click **Browse** and search for the folder you have the **Immune-response.tac_analysis_configuration** and the **Immune-response_v3.transcript.csv**, and open it (both files have to be in the same folder). You will see the files like below.



OIRRA Data Analysis

Torrent Suite Analysis – Primary Analysis



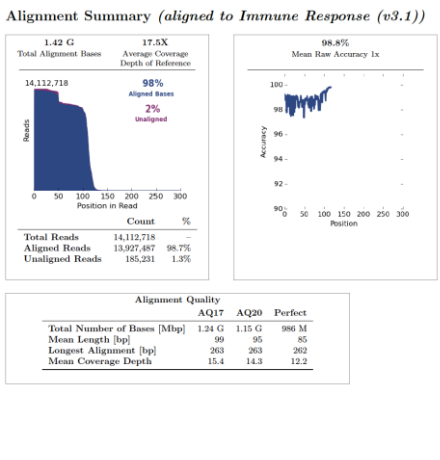
Run Report for CA_OIRRA_DL8_1_all_merged_OIRRA_001

immuneResponseRNA

Target regions: ImmuneResponse_v3.1_target_designed_20160908
Genes of interest region: ImmuneResponse_v3.1_target_designed_20160908
Read filters: Alignment length at least 50% of insert size.
Time of analysis: 2018-04-03 14:59

Barcode Name	Sample	Mapped Reads	Valid Reads	Targets >= 1 reads	Targets >= 2 reads	Targets >= 10 reads
IonCode_0101	IMM-1-R	294,591	67.00%	220	209	206
IonCode_0102	IMM-14-R	2,861,710	79.23%	348	348	346
IonCode_0103	IMM-16-R	2,106,463	83.18%	348	348	346
IonCode_0104	IMM-19-R	1,988,439	78.11%	326	322	318
IonCode_0105	IMM-20-R	3,039,529	91.56%	353	351	350
IonCode_0106	IMM-23-R	2,039,986	69.40%	341	340	339
IonCode_0107	IMM-24-R	244,087	82.75%	253	249	244
IonCode_0108	IMM-26-R	1,352,682	90.45%	304	303	299

5 items per page — 1 - 8 of 8 items



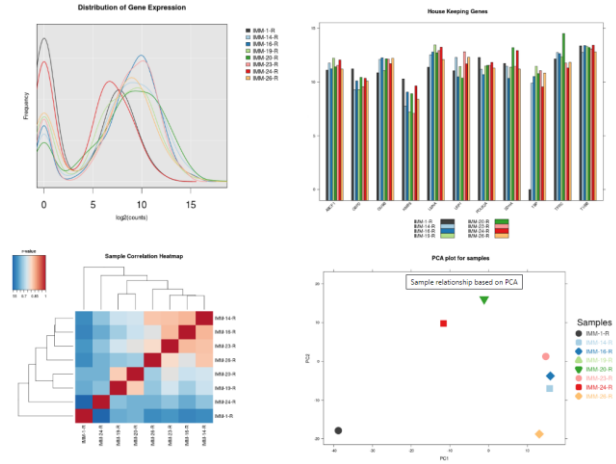
Alignment Summary *(aligned to Immune Response (v3.1))*

1.42 G — Total Alignment Bases
17.5X — Average Coverage Depth of Reference
98% Aligned Bases
2% Unaligned
98.8% — Mean Raw Accuracy 1x

	Count	%
Total Reads	14,112,718	–
Aligned Reads	13,927,487	98.7%
Unaligned Reads	185,231	1.3%

Alignment Quality

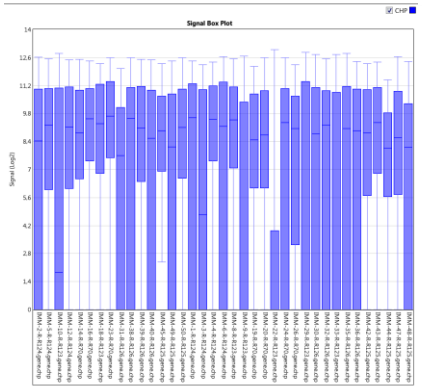
	AQ17	AQ20	Perfect
Total Number of Bases [Mbp]	1.24 G	1.15 G	986 M
Mean Length [bp]	99	95	85
Longest Alignment [bp]	263	263	262
Mean Coverage Depth	15.4	14.3	12.2



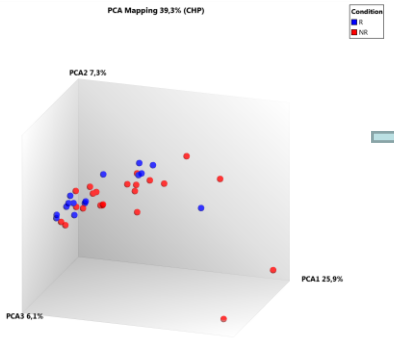
TAC analysis - Secondary Analysis

Among 37 FFPE samples only 18 showed more than 300 OIRRA detectable target genes

OIRRA all samples

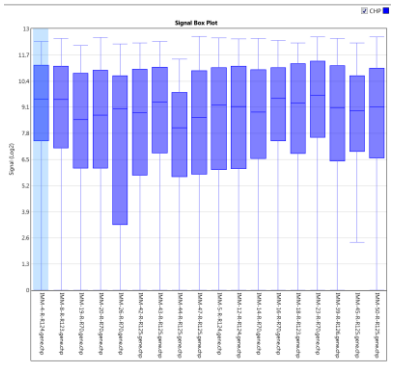


Signal Box plot

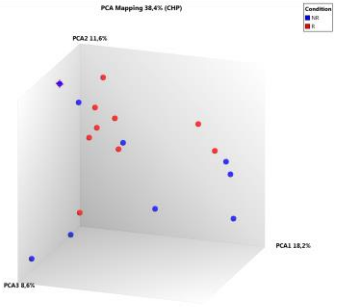


Principal Component Analysis

OIRRA > 300 targets



Signal Box plot



Principal Component Analysis

OIRRA Results

Immune Gene Expression

18 samples showed more than 300 OIRRA detectable target genes
Gene expression analysis revealed (p-value < 0.05):

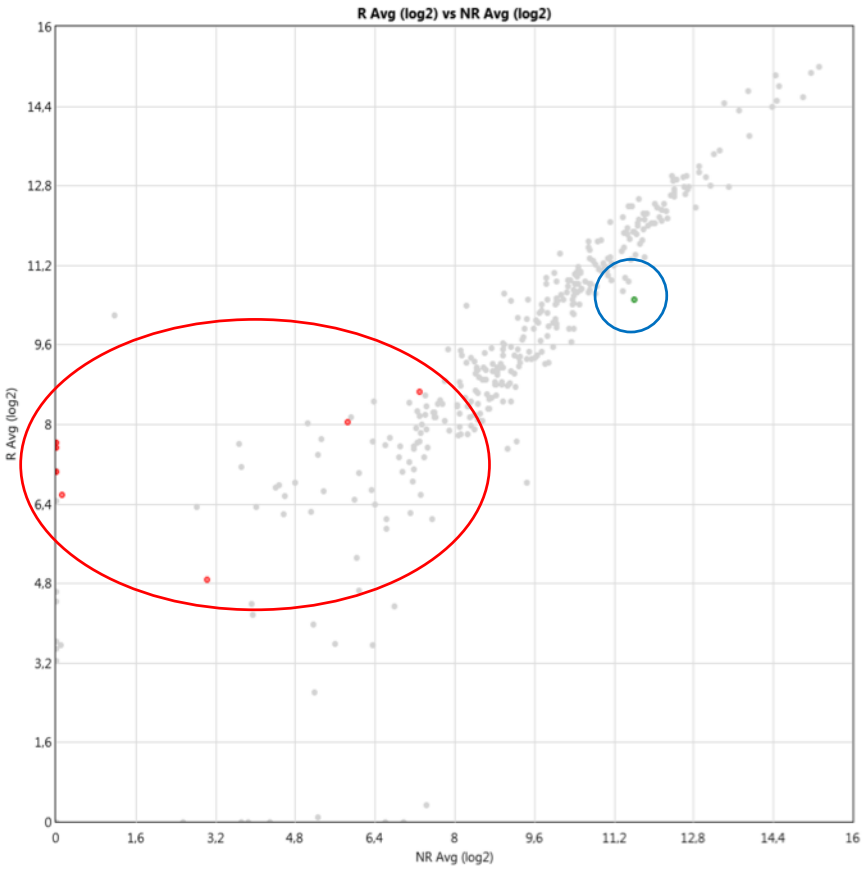
- 7 genes up-regulated

CCR2, CRTAM, FASLG, SELL, TIGIT, TNFRSF4, and TP63

- one gene down-regulated

CXCL8

onders (NR)



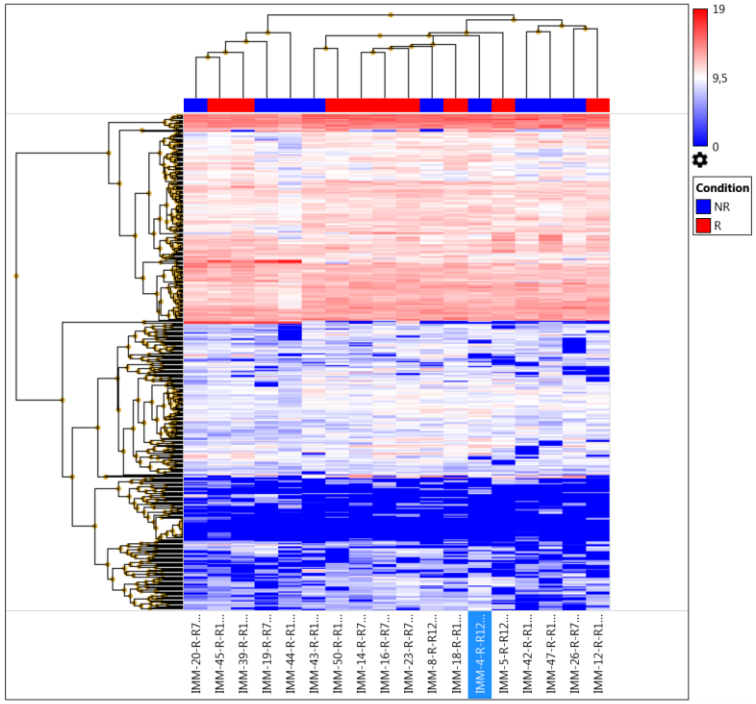
Scatter Plot R vs NR

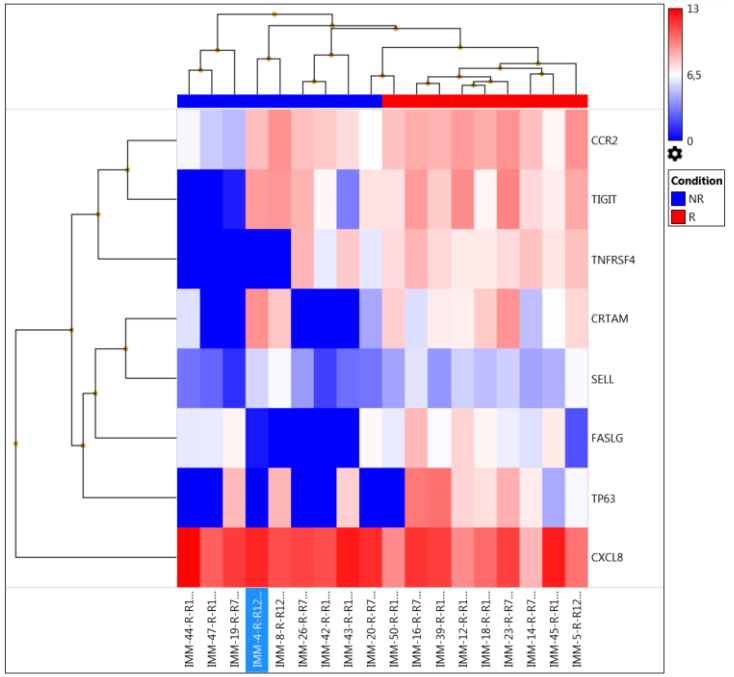
Gene Symbol			Avg (log2)	Fold Change	P-val
TNFRSF4	tumor_ne		0	185,53	0,003
CRTAM	cyt		0	132,75	0,0093
FASLG	Fa		0,13	88,02	0,0168
SELL			3,02	3,6	0,0202
CCR2			7,28	2,59	0,0245
TP63			0	197,44	0,0028
TIGIT	T_cell_i		5,83	4,66	0,0331
CXCL8			11,59	-2,13	0,0385

OIRRA Results

Immune Gene Expression

Hierarchical cluster analysis





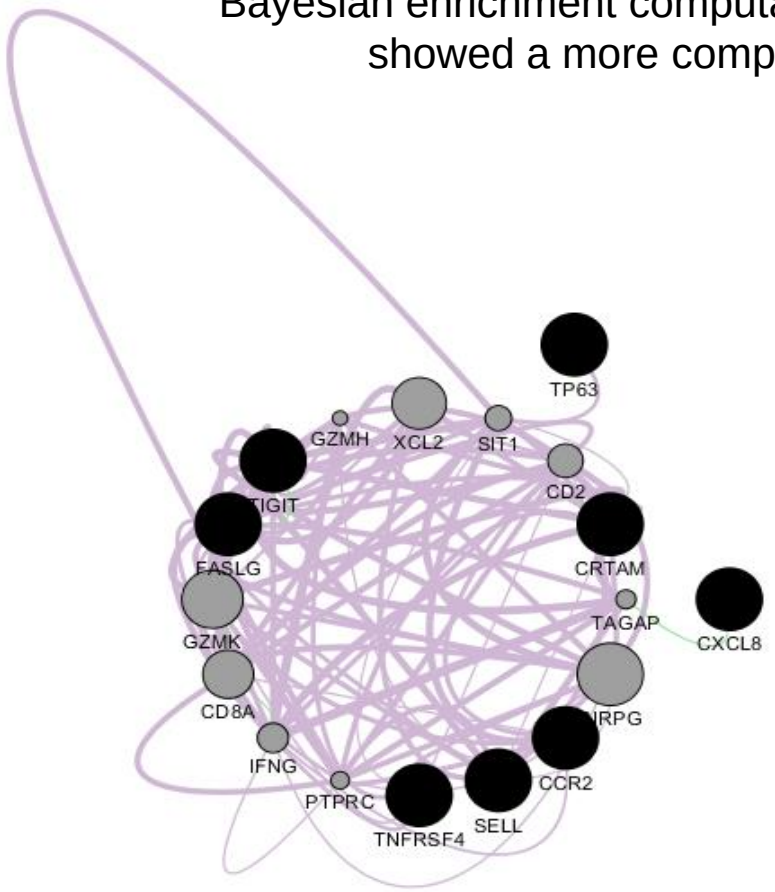
Heatmap ALL TARGETS

Heatmap gene signature TARGETS
P-val <0.05

Bayesian Computational Analysis

Gene Network based on Bayesian algorithm was performed by GeneMANIA database querying with the genes selected through mRNA expression analysis

Bayesian enrichment computational analysis of eight gene expression signature showed a more complex network which involves other 10 genes



SIRPG, GZMK, XCL2, CD8A, CD2, IFNG, SIT1, TAGAP, PTPRC and GZMH

→ correlated with different functional groups.

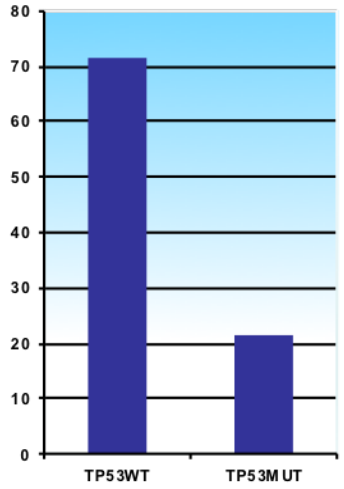
Three main immune-pathways were identified ($p < 0.01$) (**T cell activation, leucocyte activation and migration**) involving **TIGIT, TNFRSF4, CCR2 and CXCL8** genes among the gene expression signature identified

Cancer somatic mutation analysis

by Ion Torrent

Ion Ampliseq Colon and Lung Cancer Research Panel v.2

Sample type	FFPE samples
Application	Somatic mutation detection
Genes	*KRAS*, *EGFR*, *BRAF*, *PIK3CA*, *AKT1*, *ERBB2*, *PTEN*, *NRAS*, *STK11*, *MAP2K1*, *ALK*, *DDR2*, *CTNNB1*, *MET*, *TP53*, *SMAD4*, *FBX7*, *FGFR3*, *NOTCH1*, *ERBB4*, *FGFR1*, and *FGFR2*
Primer pairs, amplicon length	92 pairs of primers in a single pool 92 amplicons with an average length of 162 bp
Input DNA required	10 ng
Observed performance	Percent of amplicons with the target base coverage at 500x: >95% Average panel uniformity: 95% Average percent reads on target: 98%
Multiplexing	2 samples per Ion 314™ Chip with at least 500x sequencing coverage 8 samples per Ion 316™ Chip with at least 500x sequencing coverage 16 samples per Ion 318™ Chip with at least 500x sequencing coverage



P = 0.0027 Fisher exact test

Group	TP53^{WT}	TP53^{MUT}
% Responder	71,4% (10/14)	21,4% (6/28)

Gene mutational analysis was feasible for 42 samples

- TP53 mutations were detected in 84,6% of ICI-No responder respect to 37.5% of ICI-Responder (p<0.01)
- No STK11 mutation was found in ICI-responders, consistent with previous reports
- KRAS mutations were detected in 46% of ICI-responders respect to 16.6% of ICI-no responders

Conclusions

Our preliminary results revealed an immune response gene expression signature of 8 genes differentially expressed between ICI-responder and ICI-no responder

⬆ **CCR2, CRTAM, FASLG, SELL, TIGIT, TNFRSF4, and TP63**

⬇ **CXCL8**

Cancer systems biology analysis approach strengthen our findings identifying an immune molecular network and confirm the correlation of the gene expression signature with relevant immune regulatory functions

T cell activation, leucocyte activation and migration

If validated, our results may have an important role for the development of a robust test to select patients properly and predict immune response to enable precision immunotherapy

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Immune Gene Expression, Bayesian Network and Genetic Mutation Analysis in Advanced NSCLC Patients Treated with Immunotherapy

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Abstract ID:1361

Background

Immune checkpoint inhibitors (ICIs) have dramatically revoluzionized the therapeutic paradigm for NSCLC, but only a small subset of patients achieves durable benefit. The only adopted predictive biomarker, PD-L1 IHC testing, suffers from some limitations. A better understanding of biomarkers associated with response to ICIs is needed.

Objectives

- ✓ To assess immune gene-expression and genetic mutation profiles.
- ✓ To evaluate association with clinical response to immunotherapy in advanced NSCLC patients treated with ICI

Materials and methods

Sample collection

Thirty-seven formalin-fixed paraffin-embedded (FFPE) samples from advanced NSCLCs, treated with nivolumab starting from the 2° line of therapy, were collected. RNA/DNA were isolated using RNeasy FFPE KIT and QIAamp DNA FFPE tissue Kit, respectively, and automatically purified by QIAcube robotic workstation (Qiagen).

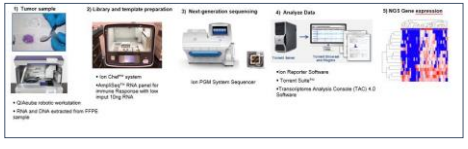
Oncomine Immuno Response Assay (OIRRA)

RNA samples were performed by RNA-Seq using the OIRRA (ThermoFisher Scientific) on Ion Torrent PGM and Transcriptome Analysis Console (TAC) v4.0 Software. This panel measures the expression level of 395 genes associated with 36 functional groups including checkpoint pathways, lymphocyte regulation and cytokine interactions.

Cancer somatic mutation

DNA samples were assessed by Ion Ampliseq Colon and Lung Cancer Research Panel v.2 that detected cancer somatic mutation of 22 genes on PGM Instrument.

NGS Workflow



Gene network analysis

Gene network analysis based on Bayesian algorithm was performed by GeneMANIA database querying with the genes selected through mRNA expression analysis..

Results

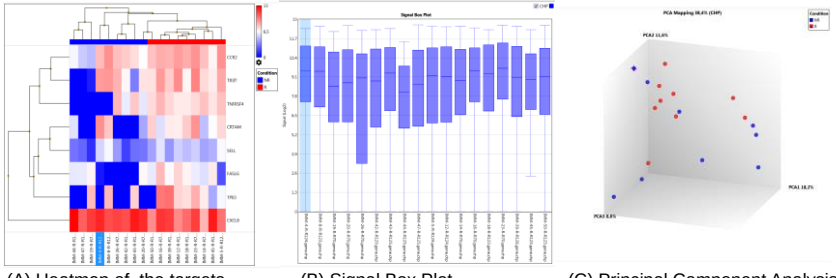
Immune Gene Expression

Among 37 FFPE samples only 18 showed more than 300 OIRRA detectable target genes. In this subgroup (Table 1), gene expression analysis revealed 7 genes (CCR2, CRTAM, FASLG, SELL, TIGIT, TNFRSF4, and TP63) up-regulated and one gene (CXCL8) down-regulated (p-value < 0.05) in ICI-responders (R) compare to ICI-no responders (NR).

Table 1

Gene Symbol	R Avg (log2)	NR Avg (log2)	Fold Change	P-val
TNFRSF4	7,54	0	185,53	0,003
CRTAM	7,05	0	132,75	0,0093
FASLG	6,59	0,13	88,02	0,0168
SELL	4,87	3,02	3,6	0,0202
CCR2	8,65	7,28	2,59	0,0245
TP63	7,63	0	197,44	0,0284
TIGIT	8,05	5,83	4,66	0,0331
CXCL8	10,5	11,59	-2,13	0,0385

Figure 1-A,B,C. User friendly analysis solution on Transcriptome Analysis Console (TAC) v4.0 Software



(A) Heatmap of the targets (B) Signal Box Plot (C) Principal Component Analysis

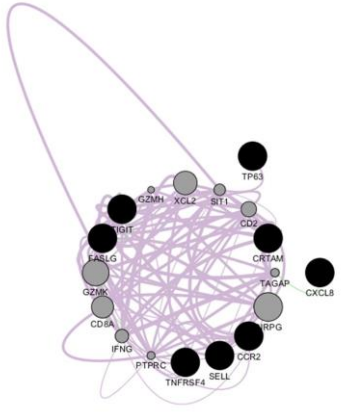
Gene mutational analysis

Gene mutational analysis was feasible for 28 samples. KRAS mutation was detected in 41% of ICI-responders respect to 12.5% of ICI-no responders. Conversely, no STK11 mutation was found in ICI-responders, consistent with previous reports.

Gene Network

Bayesian enrichment computational analysis of eight gene expression signature showed a more complex network which involves other 10 genes (SIRPG, GZMK, XCL2, CD8A, CD2, IFNG, SIT1, TAGAP, PTPRC and GZMH), correlated with different functional groups. Three main immune-pathways were identified ($p < 0.01$) (T cell activation, leucocyte activation and migration) involving TIGIT, TNFRSF4, CCR2 and CXCL8 genes among the gene expression signature identified.

Figure 2. Gene Network by Bayesian computational analysis



Conclusion

Our results revealed an immune response gene expression signature of 8 genes differentially expressed between ICI and ICI-no responders. Cancer systems biology analysis approach strengthen our findings identifying an immune molecular network and confirm the correlation of the gene expression signature with relevant immune regulatory functions. If validated, our results may have an important role for the development of a robust test to select patients properly and predict immune response to enable precision immunotherapy

References

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Acknowledgement

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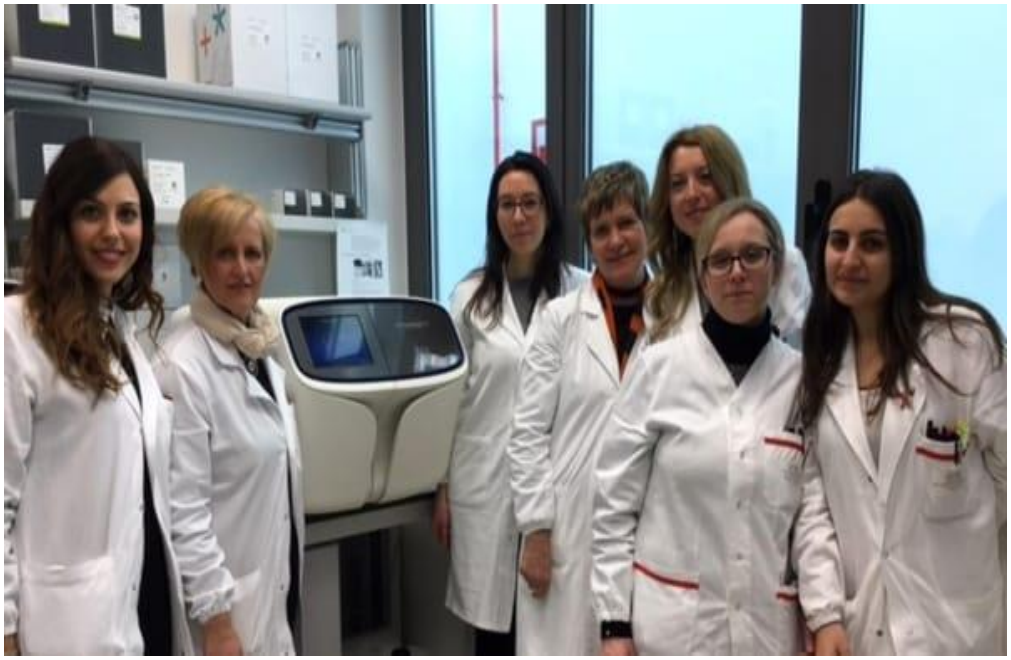
Future Perspectives

Realizing Precision Immunotherapy

- ✓ Validation of our previous findings on the predictive role of GEP on a wider retrospective and prospective population of advanced NSCLC patients
- ✓ Evaluating baseline Tumour Mutational Burden (TMB) as candidate predictive and prognostic biomarkers of immunotherapy
- ✓ Assessment of TMB using the same retrospective and prospective advanced NSCLC populations of GEP
- ✓ Assessment of Blood TMB (bTMB) on liquid biopsy at baseline and during treatment
- ✓ Gene mutational profiling in the same populations
- ✓ Analysis of GEP and TMB also on early-stage NSCLC setting as prognostic biomarkers
- ✓ Combination between RNA-Seq experiments and Computational Investigations *(Collaboration with Prof. A. Califano Systems Biology Lab at Columbia University USA and Prof. P.Valigi group at Department of Engineering, University of Perugia)*

Project submitted to AIRC IG 2019…

Development of a multidimensional approach for the understanding predictors of immune response to those patients who will derive optimal benefit from treatment



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