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INTEGRATIVE BIOLOGY

BIOSAIC RESEARCH PROGRAM

TRANSLATIONAL MEDICINE

BIOSAIC RESEARCH PROGRAM

INTEGRATED TRANSLATIONAL STUDIES IN THE MOSAIC TRIAL

PRINCIPAL INVESTIGATOR: GERCOR IRC

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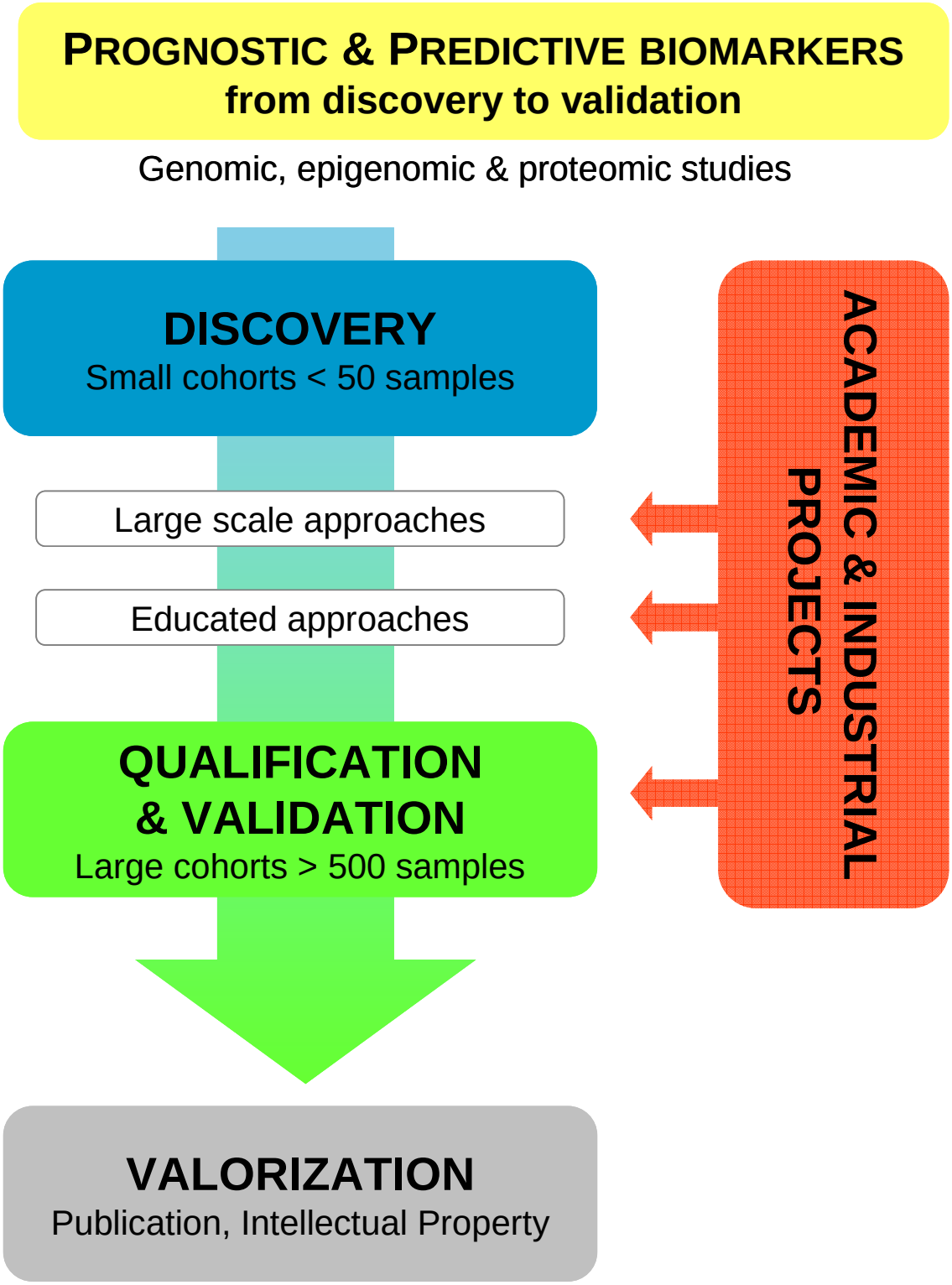

BIOSAIC RESEARCH PROGRAM

I. INTRODUCTION

I.1 ADJUVANT TREATMENT IN COLORECTAL CANCER

Colorectal carcinoma (CRC) is the third most common cancer in both men and women, and it is the second leading cause of cancer death in Western countries (American cancer society, 2009). Patients with high risk stage II and stage III colon cancer that have been resected and potentially cured (50% of all colon cancer patients) will receive an adjuvant treatment to complete the surgical intervention. Since 2004, **three trials have confirmed the benefit of adding oxaliplatin to fluoropyrimidine based adjuvant chemotherapy**:

- the MOSAIC (Multicenter International Study of Oxaliplatin/5-Fluorouracil/ Leucovorin) trial that recruited 2246 patients with stage II or III colon cancer and led to the approval of FOLFOX4 as adjuvant therapy after surgery in patients with stage III colon cancer [1].

- the NSABP (National Surgical Adjuvant Breast and Bowel Project) trial C-07 which evaluated the FLOX regimen (oxaliplatin added to a weekly bolus of 5FU/LV) in 2492 stage II and III colon cancer patients [2].

- the NO16968 trial, which evaluated the XELOX (capecitabine and oxaliplatin) regimen in 1886 patients [3].

Other trials evaluating irinotecan based chemotherapy or adding targeted therapies such as cetuximab (NO147 and PETACC-8 trials) and bevacizumab (NSABP C-08 and AVANT trials) to chemotherapy failed to demonstrate an advantage over the FOLFOX4 regimen. These recent failures in large size trials (close to 10 000 patients total for these unsuccessful trials) will certainly delay future prospective trials in this indication. For this reason and the constant improvement of personalized medicine i.e. adapting treatments to the patients that will benefit from the therapy, **we urgently need validated prognostic and predictive biomarkers in the management of adjuvant treatment in CRC**.

The **BIOSAIC Research Program** was set up to accelerate prognostic and predictive biomarkers discovery and validation in adjuvant colorectal cancer

I.2 PROGNOSTIC AND PREDICTIVE BIOMARKERS

In colon cancer, tumor extension, the number of involved lymph nodes as well as the number of examined lymph nodes, are the most potent prognostic factors [4-7]. A brief review of the literature show that addition of the genetic/epigenetic modification

phenotypes (CIN: chromosomal instability vs CIMP: CpG island methylator phenotype), loss of DNA mismatch repair (MMR) gene expression (MSI: microsatellite instability) [5, 8-10], KRAS and BRAF mutations [11, 12], high p53, VEGF and CXCR4 expression [10, 13, 14], loss of SMAD4, Cbx7 and 18q locus heterozygosity [15, 16], gene signature (Oncotype DX) [17], may help to better define the patient sub-populations that may benefit from adjuvant therapy. A recent analysis of the NASPB C-07 trial showed that BRAF mutated tumors were associated with poor overall survival (OS) and poor survival after recurrence (SAR) but not with disease free survival (DFS). BRAF mutations were also associated with MMR-deficient tumors but, in contrast to BRAF mutations, MMR-deficient tumors were associated with improved DFS, OS and SAR. MMR-proficient and BRAF mutant tumors displayed the worst prognosis. All the other mutations analyzed, KRAS, NRAS, MET and PIK3CA were not associated with DFS, OS or SAR. As well, neither MMR, nor mutations were associated with oxaliplatin benefit [18].

Epigenetic alterations, such as gene methylation, have also been reported to be involved in the CRC pathogenesis [19]. In a recent study among 4 different cohorts of mCRC or primary rectal cancer undergoing surgery or chemotherapy, authors identified hypermethylated TFAP2E in 39% of mCRC and in 45% of primary rectal cancer. In all groups, hypermethylation was strongly associated with a lack of response to chemotherapy (FOLFIRI +/- cetuximab, FOLFOX) or chemoradiation ($p<0.001$) [20]. As a target of TFAP2E, DKK4 overexpression has previously been implicated in fluorouracil resistance in colon cancer [21-22]. Authors suggest that TFAP2E gene inhibition through hypermethylation, and thus DKK4 overexpression may be potential negative predictive biomarkers of chemotherapy resistance in general.

A recent Korean retrospective study among 322 patients with stage II/III colorectal cancer who received FOLFOX as adjuvant chemotherapy showed that the concurrent methylation of NEUROG1 and CDKN2A (p16) genes was associated with poor (DFS) [23].

A comprehensive study of genetic and epigenetic modifications in colon and rectal cancer has been recently published by the Cancer Genome Atlas Network [24]. However, due to its recency this study lacks the possibility to correlate the genetic and epigenetic observations with response or survival parameters.

The goal of the **BIOSAIC Research Program** is to uncover and validate new biomarkers as part of new or running translational discovery programs from academic or industry

The following work packages (WP) are prerequisite to initiate any study projects. Discovery, qualification and validation studies need to be evaluated and prioritized to ensure their realization and optimized their potentials.

II. BIOSAIC LIBRARIES AND PRELIMINARY EVALUATIONS

II.1 WP1: CONSTITUTION OF A TISSUE MICROARRAY (TMA) LIBRARY

To this date, tissue microarrays have been set up from 800 parrafin embedded tumor samples. This work has been realized in the pathology department of Saint-Antoine hospital under the supervision of Pr FLEJOU.

II.2 WP2: CONSTITUTION OF DNA AND CDNA LIBRARIES

In order to allow the implementation of research projects on the MOSAIC trial tumor blocks, it is necessary to extract genomic DNA as well as RNA converted in cDNA to implement libraries from which projects will be executed. This work will be done by AAREC (Association d'Aide à la Recherche et l'Enseignement en Cancérologie) in Beaujon hospital .

DNA extraction
The paraffin-embedded tissues will be sliced and deparaffinized. The DNA extraction will be automatically done using QIAcube (QIAGEN) after the manufacturer instructions, using QiaAmp DNA FFPE Tissues kit (Qiagen).

RNA extraction and cDNA conversion
The paraffin-embedded tissues will be sliced and deparaffinized. The RNA extraction will be automatically done using QIAcube (QIAGEN) after the manufacturer instructions, using RNAeasy FFPE Tissues kit (Qiagen). Extracted RNA will then be converted into cDNA by reverse transcription.

Storage & availability
DNA and cDNA libraries will be stored in Beaujon hospital and available upon request.

II.3 WP3: PRELIMINARY EVALUATIONS

All tumors will be analyzed for their microsatellite status using immunohistochemistry against MSH1, MLH2, MSH6 and PMS2. This work will be realized by Dr Alex Duval

and Pr Jean François Flejou. Mutation analyses of different genes (KRAS, BRAF, NRAS, PI3KCA…) will be realized through academic platforms in either St Antoine, Bichat or Beaujon (AAREC) hospitals. In collaboration with the NSABP, we will first start with identification of the BRAF mutations, in order for the NSABP to realize a meta-analysis on the NSABP-C07 and MOSAIC trials and definitively validate MMR/BRAF patients' prognostics.

III. DISCOVERY STUDIES

II.1 STUDY 1: EPIGENETIC MODIFICATION AS POTENTIAL PROGNOSTIC BIOMARKERS AND PREDICTIVE BIOMARKERS OF CHEMOTHERAPY EFFICACY

Objectives: To identify epigenetic modification differences between early and late recurrence among the two arms and to identify epigenetic modification differences that would correlate with response to chemotherapy especially oxaliplatin.

Methods: We plan to use the Infinium Methylation Assay from Illumina on 40 samples, 20 from each arm (5-FU and FOLFOX4), representative of patients with early and late cancer recurrence. This experimentation will be done through research collaboration.

Estimated delay: 6 months

II.2 STUDY 2: GENE EXPRESION TO UNCOVER POTENTIAL PROGNOSTIC BIOMARKERS AND PREDICTIVE BIOMARKERS OF CHEMOTHERAPY EFFICACY

Objectives: To identify gene that may be differentially expressed between early and late recurrence patients groups among the two arms and that may correlate with response to chemotherapy especially oxaliplatin. The second objective is to identify genetic modification differences in the above groups.

Methods: Gene expression and sequencing screening will be done using the RNAseq technology that allows deep sequencing and quantitative evaluation of gene expression. This experimentation will be done through research collaboration.

Estimated delay: 6 months

II.3 STUDY 3: MUTATION SCREENING TO UNCOVER POTENTIAL PROGNOSTIC BIOMARKERS AND PREDICTIVE BIOMARKERS OF CHEMOTHERAPY EFFICACY

Objectives: To identify genetic mutations that may discriminate early and late recurrence among the two arms and to identify epigenetic modification differences that would correlate with response to chemotherapy especially oxaliplatin.

Methods: Mutations screening will be done in an educated guess manner on a predefine set of genes. We plan to use the Ion AmpliSeq™ Ready-to-Use or Custom Panels (Life Technologies).

Estimated delay: 3 months

III. QUALIFICATION & VALIDATION STUDIES

III.1 STUDY 4: VALIDATION OF TFAP2E METHYLATION AS A PREDICTIVE BIOMARKER OF RESISTANCE TO CHEMOTHERAPY

The study is aimed at confirming that the hypermethylation status of TFAP2E can be predictive of chemotherapy resistance.

Objectives: To test if the methylation status of TFAP2E, p16, NEUROG1, LIFR and LINE-1, as well as the MSI status, correlate with 3-year DFS, OS, treatment and any pathological data (stage, number of examined/positive lymph nodes, differentiation…).

Methods: DNA samples from the MOSAIC trial will be treated with the EpiTect Plus DNA Bisulfite Kit (QIAGEN) to convert unmethylated cytosine into uracil while methylated cytosines in CpG sequences remain unaffected. DNA methylation of the proposed genes will be analyzed using the PyroMark Q24 following the manufacturer instructions (QIAGEN).

Estimated delay: 4 months

V. TIMELINES AND BUDGET

V.1 TIMELINES

	YEAR 1				YEAR 2			
	T1	T2	T3	T4	T1	T2	T3	T4
WP1: TMA library								
WP2: DNA & cDNA libraries								
WP3: Preliminary experiments								
Discovery studies								
STUDY 1: Epigenetic screening								
STUDY 2: RNAseq								
STUDY 3: Mutation screening								
Qualification & Validation studies								
STUDY 4: TFAP2E methylation								
STUDY 5: Biomarker evaluation from study 1, 2 & 3								

V.2 BUDGET

	Cost/item	Current fundings
WP1: TMA library		
Staff (100% FTE, 6 months)		
Total WP1	**0 €**	Genomic Health
WP2: DNA & cDNA libraries		
Staff (50% FTE, 2 months)	4 000 €	
DNA extraction (800 samples)	5 400 €	
RNA extraction and cDNA conversion (800 samples)	10 600 €	
Total WP2	**20 000 €**	None → ARCAD
WP3: Preliminary experiments		
Staff (100% FTE, 3 months)	10 000 €	
BRAF mutations (800 samples)	30 000 €	
Total WP3	**40 000 €**	None → ARCAD

STUDY 1: Epigenetic screening		
Research collaboration	~ 30 000 €	None
Total Study 1	**~ 30 000 €**	None
STUDY 2: RNAseq		
Research collaboration	~ 30 000 €	None
Total Study 2	**~ 30 000 €**	None
STUDY 3: Mutation analysis		
Research services	6 – 15 000 €	None
Total Study 3	**6 – 15 000 €**	None
STUDY 4: TFAP2E methylation		
Staff (25% FTE, 4 months)	4 000 €	None
FFPE conversion (800 samples)	5 600 €	None
CpG methylation assay (2 genes in duplicate on 800 samples)*	40 000 €	None
Total Study 4	**49 600 €**	None

* cost may be reduced with statistical hypothesis

Request for ARCAD support

This is a step by step project. The first step consists in WP1, WP2 and WP3. The suggested studies (Study 1-4) may be realized through ARCAD support or other funding and will be re-submitted to the ARCAD scientific board later on.

STEP1
WP1: done through Genomic health support
WP2: 20 000 €
WP3: 40 000 €

Total requested: 60 000 €

APPENDIX I. REFERENCES

[1] T. André, C. Boni, L. Mounedji-Boudiaf et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer: The Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) Investigators. N Engl J Med, 350 (2004), pp. 2343–2351.

[2] J.P. Kuebler, H.S. Wieand, M.J. O'Connell et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon: results from NSABP C-07. J Clin Oncol, 25 (2007), pp. 2198–2204.

[3] D.G. Haller, J. Tabernero, J. Maroun et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. J Clin Oncol, 29 (2011), pp. 1465–1471.

[4] American Joint Committee on Cancer. AJCC Cancer Staging Manual (7th edition), 978-0-387-88440-0Springer, New York, NY (2010).

[5] J.B. Bachet, P. Laurent-Puig, A. de Gramont et al. Microsatellite status and adjuvant chemotherapy in patients with stage II colon cancer. Curr Colorectal Cancer Rep, 6 (2010), pp. 148–157.

[6] P.M. Johnson, G.A. Porter, R. Ricciardi et al. Increasing negative lymph node count is independently associated with improved long-term survival in stage IIIB and IIIC colon cancer. J Clin Oncol, 24 (2006), pp. 3570–3575.

[7] A.C. Berger, E.R. Sigurdson, T. LeVoyer et al. Colon cancer survival is associated with decreasing ratio of metastatic to examined lymph nodes. J Clin Oncol, 23 (2005), pp. 8706–8712.

[8] D.J. Sargent, S. Marsoni, G. Monges et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. J Clin Oncol, 28 (2010), pp. 3219–3226.

[9] A.D. Roth, S. Tejpar, P. Yan et al. Molecular markers in colon cancer have a stage specific prognostic value: Results of the translational study on the PETACC 3 - EORTC 40993 -SAKK 60-00 trial. J Clin Oncol, 27 (suppl 15S) (2009) 4002.

[10] Zaanan A, Cuilliere-Dartigues P, Guilloux A et al. Impact of p53 expression and microsatellite instability on stage III colon cancer disease-free survival in patients treated by 5-fluorouracil and leucovorin with or without oxaliplatin. Ann Oncol. 2010 Apr;21(4):772-80.

[11] S.R. Alberts, D.J. Sargent, T.C. Smyrrk et al. Adjuvant mFOLFOX6 with or without cetuximab in KRAS wild-type patients with resected stage III colon cancer: results from NCCTG intergroup phase III trial NO147. J Clin Oncol, 28 (suppl 18S) (2010), p. 959s

[12] A.D. Roth, S. Tejpar, M. Delorenzi et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the Translational Study on the PETACC-3, EORTC 40993, SAKK 60-00 Trial. J Clin Oncol, 28 (2010), pp. 466–474.

[13] J. Kim, H. Takeuchi, ST Lam, et al. Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. J Clin Oncol. 2005 Apr 20;23(12):2744-53.

[14] A. Ottaiano, R. Franco, A. Aiello Talamanca et al. Overexpression of both CXC chemokine receptor 4 and vascular endothelial growth factor proteins predicts early distant relapse in stage II-III colorectal cancer patients. Clin Cancer Res. 2006 May 1;12(9):2795-803.

[15] P.L. Pallante, L. Terracciano, V. Carafa et al. The loss of the CBX7 gene expression represents an adverse prognostic marker for survival of colon carcinoma patients. Eur J Cancer, 46 (2010), pp. 2304–2313.

[16] T. Watanabe, T.T. Wu, P.J. Catalano et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. N Engl J Med, 344 (2001), pp. 1196–1206

[17] M.J. O'Connell, I. Lavery, G. Yothers et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. J Clin Oncol, 28 (2010), pp. 3937–3944.

[18] P.G. Gavin, L.H. Colangelo, D. Fumagalli et al. Mutation profiling and microsatellite instability in stage II and III colon cancer: an assessment of their prognostic and oxaliplatin predictive value. Clin Cancer Res (2012); 18(23).

[19] M. Esteller. Epigenetics in cancer. N Engl J Med. 2008 Mar 13;358(11):1148-59.

[20] M.P.A Ebert, M. Tänzer, B. Balluff et al. TFAP2E-DKK4 and chemoresistance in colorectal cancer. N Engl J Med, 366 (2012), pp. 44-53.

[21] Y. XI, G. Nakajima, J.C. Schmitz et al. Multi-level gene expression profiles affected by thymidylate synthase and 5-fluorouracil in colon cancer. BMC Genomics 7 (2006), pp.68

[22] Y. XI, A. Formentini, G. Nakajima et al. Validation of biomarkers associated with 5-fluorouracil and thymidylate synthase in colorectal cancer. Oncol Rep 19 (2008), pp. 257-62.

[23] S-W Han, H-J Lee, J-M Bae et al. Methylation and microsatellite status and recurrence following adjuvant FOLFOX in colorectal cancer. Int. J. Cancer (2013), 132: 2209-2216.

[24] The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012 Jul 18;487(7407):330-7.