

# Sensitivity and Resistance to Regorafenib Therapy in Advanced Colorectal Cancer: *ctDNA Monitoring and Molecular Mechanisms*

Thesis submitted by **Pashalina Kehagias**

in fulfilment of the requirements of the PhD Degree in

Biomedical and Pharmaceutical Sciences

(« Docteur en Sciences Biomédicales et Pharmaceutiques »)

*Academic Year 2019-2020*

Supervisor : Professor **Alain HENDLISZ**,

Digestive Oncology

Department of Medical Oncology

Institut Jules Bordet



Fondation  
Rose et Jean Hoguet

# TABLE OF CONTENTS

---

## ACKNOWLEDGMENTS

## ABSTRACT

## TABLE OF CONTENTS

LIST OF ABBREVIATIONS.....	11
INTRODUCTION.....	13
1.1    Colorectal Cancer.....	13
1.1.1    Screening and Diagnosis.....	13
1.1.2    CRC Origin, Progression and Staging.....	13
1.1.3    Molecular Pathology of CRCs.....	16
1.1.3.1    CRC Carcinogenesis.....	16
1.1.3.2    Pathways Resulting to Genomic instability.....	16
1.1.3.3    Altered Signaling Pathways in CRC.....	17
1.1.3.4    Genetics and Genomics in CRC.....	20
1.1.3.5    CRC Molecular Subtypes.....	22
1.2    CRC Treatment Options.....	24
1.2.1    General Overview.....	24
1.2.2    Targeted Therapies in CRC.....	24
1.2.3    Evaluation of Treatment efficacy.....	26
1.2.4    The multi-kinase inhibitor Regorafenib.....	26
1.2.4.1    Regorafenib Mechanism of Action.....	27
1.2.4.2    Pharmaceutical Properties and Pharmacodynamic Effects.....	28
1.2.4.3    Regorafenib efficacy and side effects in CRC.....	28
1.2.4.4    Tumor Response Assessment under Regorafenib.....	28

1.3	Biomarkers for CRC.....	30
1.3.1	RAS and RAF Mutational Status.....	30
1.3.2	Carcinoembryogenic Antigen (CEA) .....	30
1.3.3	Carbohydrate Antigen (CA19-9).....	30
1.3.4	Novel Reliable Biomarkers.....	31
1.4	Liquid Biopsy.....	32
1.4.1	Overview.....	32
1.4.2	Circulating Cell-Free DNA.....	33
	1.4.2.1 Physiopathology.....	33
	1.4.2.2 Circulating Tumor DNA.....	34
1.4.3	Clinical applications in Oncology.....	34
	1.4.3.1 Screening and Diagnosis.....	34
	1.4.3.2 Diagnosis of Residual disease.....	35
	1.4.3.3 Evaluation of treatment efficacy.....	36
1.4.4	Tissue versus Liquid Biopsy.....	37
	1.4.4.1 Mutation Status of Tumor Tissue.....	37
	1.4.4.2 Clonal Heterogeneity.....	37
	1.4.4.3 Acquired resistance mechanisms.....	38
1.5	Targeted Therapy Resistance.....	39
1.5.1	Multidrug Resistance in Targeted Therapy.....	39
1.5.2	Tumor Cell Plasticity under Targeted Therapy.....	40
	1.5.2.1 Cellular Senescence.....	40
	1.5.2.2 Epithelial-Mesenchymal Transition (EMT) .....	46
	1.5.2.3 EMT and Acquisition of Senescence-like Properties.....	48

<b>THESIS AIMS.....</b>	<b>49</b>
2.1    Objectives of RegARD-C Translational Research Study.....	49
2.2    Objectives of Regorafenib Mechanistic Study in CRC Cell Lines.....	50
<b>MATERIALS AND METHODS.....</b>	<b>51</b>
3.1    RegARD-C Translational Research Study.....	51
3.1.1    Study Design and Populations.....	51
3.1.2 <sup>18</sup> Fluoro-DeoxyGlucose (FDG) PET-CT Imaging.....	52
3.1.3    Sample Collection and Plasma Isolation.....	52
3.1.4    DNA Extraction and Quantification.....	53
3.1.5    Targeted Gene Sequencing.....	53
3.1.6    Droplet Digital PCR and Data Analysis.....	54
3.1.7    Prime PCR TM ddPCR TM Mutation Detection Assays Optimization.....	56
3.1.8    CEA and CA19-9 Evaluation.....	58
3.1.9    Statistical analysis.....	58
3.2    Regorafenib Mechanistic Study in CRC Cell Lines.....	60
3.2.1    Cell Lines and Cell Culture Conditions.....	60
3.2.2    Reagent.....	60
3.2.3    Cell Viability Assay.....	60
3.2.4    Short and Long-term Regorafenib Exposure.....	61
3.2.5    Detection and Measurement of β-galactosidase Activity.....	61
3.2.6    Cell Cycle Analysis.....	62
3.2.7    Cell Migration Assay.....	62
3.2.8    RNA Isolation and Reverse Transcriptase qPCR.....	63
3.2.9    Reverse Phase Protein Array (RPPA) Profiling.....	64
3.2.10    Western Blot Analysis.....	65

3.2.11 Cytokine Array Profiling.....	66
3.2.12 Enzyme Linked Immunosorbent Assay (ELISA) .....	67
3.2.13 ImmunoHistoChemistry (IHC) .....	68
3.2.14 Statistical Analysis.....	68
<b>R E S U L T S.....</b>	<b>69</b>
4.1 RegARD-C Translational Research Study.....	69
4.1.1 Study Population.....	69
4.1.2 Exploratory Study on Novel Biomarkers .....	69
4.1.2.1 Targeted Gene Sequencing Analysis on Tissue and Plasma Samples.....	71
4.1.2.2 Prognostic Value of BL cfDNA.....	74
4.1.2.3 CtDNA Monitoring and Data Interpretation.....	74
4.1.2.4 Prognostic Value of BL-D14 ctDNA Dynamics.....	77
4.1.3 Extended Study on Conventional and Novel Biomarkers .....	79
4.1.3.1 Assessment of Conventional Biomarkers.....	79
4.1.3.2 Assessment of Novel Biomarkers.....	82
4.1.3.3 Univariate and Multivariate Analyzes including Clinical Parameters.....	95
4.1.4 Validation of Early Regorafenib Response Assessment in an Independent Cohor....	97
4.1.4.1 Study Population.....	97
4.1.4.2 Prognostic Value of BL cfDNA.....	98
4.1.4.3 Prognostic Value of BL-D14 ctDNA Dynamics.....	98
4.2 Regorafenib Mechanistic Study in CRC Cell Lines.....	99
4.2.1 Regorafenib Effect on Cell Survival.....	99
4.2.2 Approaches to uncover resistance mechanisms.....	99
4.2.3 Regorafenib Induces Senescence-like Phenotype.....	101
4.2.3.1 Cell Morphology change under regorafenib.....	101

4.2.3.2 SA- $\beta$ -galactosidase Detection and Activity under Regorafenib.....	101
4.2.3.3 Regorafenib Effect on Cell Cycle.....	105
4.2.3.4 Identification of Key Regulators of Senescence-like Phenotype.....	108
4.2.3.5 Regorafenib-Induced Senescence Associated Secretory Profile (SASP).....	110
4.2.4 Regorafenib Promotes Epithelial-to-Mesenchymal Transition (EMT).....	111
4.2.4.1 Regorafenib Effect on Cell Migration.....	111
4.2.4.2 Identification of EMT Key Regulators.....	112
4.2.5 Multidrug Resistance Evaluation In Colorectal Cancer.....	115
<b>DISCUSSION.....</b>	<b>118</b>
5.1 RegARD-C Translational Research Study .....	118
5.2 Regorafenib Mechanistic Study in CRC Cell Lines.....	125
<b>CONCLUSION AND PERSPECTIVES.....</b>	<b>133</b>
<b>REFERENCES.....</b>	<b>136</b>
<b>SUPPLEMENTARY DATA.....</b>	<b>151</b>
<b>SCIENTIFIC PUBLICATIONS.....</b>	<b>162</b>