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WARBURG EFFECT AND TP53 MUTATIONAL STATUS IN GASTRIC CANCER PATIENTS UNDER RAMUCIRUMAB-BASED THERAPY

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ABSTRACT

Gastric cancer is one of the most common cancers worldwide and ranks second in cancer-related deaths. To improve the survival rate, several studies have elucidated molecular mechanisms of gastric cancer and identified biomarkers predicting prognosis and response to treatment. Cancer cells exhibit altered glucose metabolism, known as the Warburg effect, characterized by the increased uptake of glucose and rates of aerobic glycolysis even under adequate oxygen levels, leading to lactate accumulation and finally promoting tumor invasion, angiogenesis, immune escape and resistance.

Overexpression of key effectors of the Warburg effect, as well as *TP53* mutational status, are associated with poor prognosis in cancer but limited data are available in gastric cancer patients under anti-angiogenic therapy.

So we have set up two studies: in the first study we investigated whether a positive glycolytic profile in gastric adenocarcinomas might be associated with unfavorable outcomes in patients treated with anticancer systemic therapy, including the anti-angiogenic Ramucirumab. For this purpose, we analyzed the mRNA expression of five key Warburg effect genes, such as *GLUT1, HK1, HK2, PKM2* and *LDHA* in 40 metastatic gastric adenocarcinoma patients under Paclitaxel-Ramucirumab (PR) treatment. We observed that patients with a positive glycolytic profile were related with worse progression-free and overall survival times.

In the second study we evaluated the possible predictive impact of *TP53* mutations on PR therapy compared to standard chemotherapy. On the basis of the residual transcriptional activity score (RTAS), the *TP53* mutations found were classified in $TP53_{active}$ and $TP53_{inactive}$. Therefore we observed that $TP53_{inactive}$ mutations differentially affect survival outcomes depending on the anti-cancer regimen, in particular PR-treated patients displaying $TP53_{inactive}$ mutations showed a better overall survival respect to patients carrying $TP53_{active}$ mutations.

Taken together these findings show that both the glycolytic competence of gastric cancer cells and the $TP53_{inactive}$ mutations may be valuable biomarkers to identify patients with greatest benefit from the anti-angiogenic PR therapy.

This Thesis is based on the following original research articles:

I. Ruzzo A, Graziano F, **Bagaloni I**, Di Bartolomeo M, Prisciandaro M, Aprile G, Ongaro E, Vincenzi B, Perrone G, Santini D, Fornaro L, Vivaldi C, Tomasello G, Loupakis F, Lonardi S, Fassan M, Valmasoni M, Sarti D, Lorenzini P, Catalano V, Bisonni R, Del Prete M, Collina G, Magnani M. Glycolytic competence in gastric adenocarcinomas negatively impacts survival outcomes of patients treated with salvage paclitaxel-ramucirumab. Gastric Cancer 2020;23(6):1064-1074. doi: 10.1007/s10120-020-01078-0

II. Graziano F, Fischer NW, **Bagaloni I**, Di Bartolomeo M, Lonardi S, Vincenzi B, Perrone G, Fornaro L, Ongaro E, Aprile G, Bisonni R, Prisciandaro M, Malkin D, Gariépy J, Fassan M, Loupakis F, Sarti D, Del Prete M, Catalano V, Alessandroni P, Magnani M, Ruzzo A. TP53 Mutation Analysis in Gastric Cancer and Clinical Outcomes of Patients with Metastatic Disease Treated with Ramucirumab/Paclitaxel or Standard Chemotherapy. Cancers (Basel) 2020;12(8):2049. doi: 10.3390/cancers12082049

CONTENT

INTRODUCTION

1.1 GASTRIC CANCER EPIDEMIOLOGY

Gastric cancer (GC) is an important health problem, being the fifth most common cancer and the second leading cause of cancer death worldwide (Fig.1). [1]

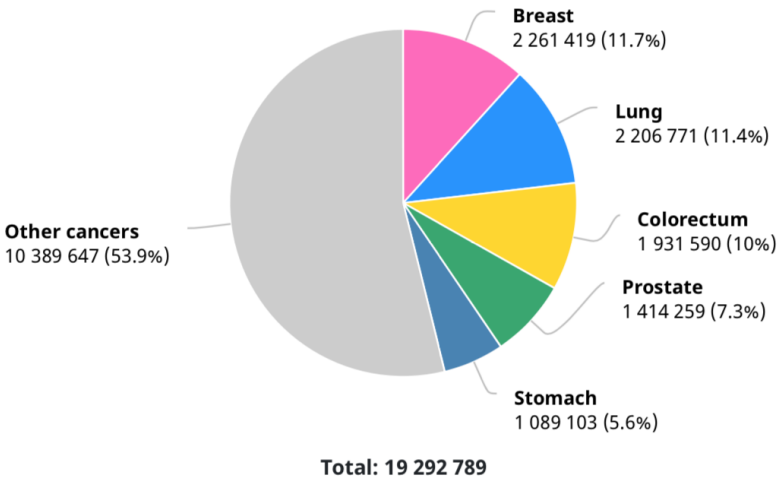

Figure.1 Number of new cases in 2020, both sexes, all ages worldwide. [1]

Despite a decline in incidence and mortality and despite important advances in the understanding of epidemiology, pathology, molecular mechanisms, therapeutic options and strategies, the GC burden remains high.

Over 140,000 cases, with over 100,000 related deaths, of GC were estimated in Europe in 2018 . Excluding skin cancers, overall GC represents 4% of all neoplasms in both sexes and it is in sixth place in terms of incidence (13.7 cases/100,000 individuals in both sexes) and in fourth place in terms of mortality (10.3 deaths/100,000) in Europe. Stomach cancer represents the fifth incident cancer among men (19.5 cases/100,000) and the seventh among women (9.3 cases/100,000). There is a considerable geographical variation in Europe, which makes it possible to distinguish countries with a higher incidence such as Portugal, Estonia, Lithuania, Slovenia (incidence around 20 cases/100,000), countries with lower incidence such as the United Kingdom, France, Norway, Sweden (incidence of less than 10 cases/100,000) and countries with intermediate incidence such as Italy, Spain, Romania, Slovakia (incidence between 10 and 20 cases/100,000). The incidence also varies with age and reaches its peak in the seventh decade. In

addition to the overall reduction in incidence, a relative increase in primitive forms with a proximal site is observed, in particular for those at the gastroesophageal junction. [2]

In Italy, overall GC represents about 4% of all cancers in both sexes, is eighth in incidence in men (4% of all cancers in men) and in ninth place in women (3% of all cancers in females). With about 5% of deaths, GC occupies the fifth place in both sexes in terms of mortality in Italy (Tab.1).

Rango	Maschi			Femmine		
	Età			Età		
	0-49	50-69	70+	0-49	50-69	70+
Totale casi incidenti	100% n=15.829	100% n=76.201	100% n=102.724	100% n=29.918	100% n=66.446	100% n=85.493
1°	Testicolo 12%	Prostata 22%	Prostata 20%	Mammella 41%	Mammella 35%	Mammella 22%
2°	Melanomi 10%	Polmone 14%	Polmone 17%	Tiroide 15%	Colon-retto 11%	Colon-retto 16%
3°	LNH 8%	Colon-retto 12%	Colon-retto 14%	Melanomi 8%	Utero (corpo) 7%	Polmone 8%
4°	Tiroide 8%	Vescica* 9%	Vescica* 11%	Colon-retto 4%	Polmone 7%	Pancreas 6%
5°	Colon-retto 7%	VADS** 5%	Stomaco 5%	Utero cervice 4%	Tiroide 5%	Stomaco 5%

Tabella 1. Five most frequent cancers (excluding non-melanoma skin cancers) as percentage of total incident cancers estimated for 2020, by gender and age group. AIRTUM Pool, 2008-2016. The data presented are not the result of estimates but are real cases provided by the registers for the years indicated * Infiltrating and non-infiltrating neoplasms are included

** VADS (Superior Aero Digestive Tracts), include the following sites: tongue, mouth, oropharynx, nasopharynx, hypopharynx, pharynx, larynx (I numeri del cancro in Italia 2020)

In Italy GC has occurred a constant reduction in incidence and mortality in both men and women. There is also a considerable geographical variation in incidence in Italy. In fact, we can distinguish areas with a higher (central regions, incidence 37 cases/100,000 in men and 21 cases/100,000 in women), intermediate (northern regions, incidence 34 cases/100,000 in men and 17 cases/100,000 in women) and low incidence (southern regions, incidence 24 cases/100,000 in men and 13 cases/100,000 in women). For cases arising in Italy in the period 2005-2009, the 5-year survival is around 32% (31% in males and 34% in females). [2]

1.2 GASTRIC CANCER AETIOLOGY

H. pylori infection is the most relevant cause of sporadic GC. During the chronic inflammation induced by *H. pylori* infection, altered cell proliferation, apoptosis and tumor suppressor genes epigenetic modifications may occur, which could eventually lead to inflammation-associated carcinogenesis. Some patients with persistent *H. pylori* infection develop gastric atrophy followed by intestinal metaplasia, which might evolve into dysplasia and adenocarcinoma. Another pathogen associated with gastric cancer is the Epstein-Barr virus, which influences gastric cancer progression in 10% of cases, but its role in carcinogenesis is still unclear. [3]

Inherited mutations of certain genes, such as the Glutathione S-transferase M1 (*GSTM1*), Cadherin 1 (CDH1) and Adenomatous Polyposis Coli (APC) have been found to increase the risk of GC. About 10% of GC cases occur in families and between 1% and 3% are due to inherited genetic syndromes such as hereditary diffuse GC, gastric adenocarcinoma, proximal polyposis of the stomach, and familial intestinal GC. Environmental factors such as diet, exercise and chemical exposure have important causal roles in GC. Low intake of fruits and vegetables and high consumption of salts, processed and heavily inflammatory foods (such as meat), as well as smoking, have been associated with increased risk of GC. Obesity is an especially strong predisposing factor for GC, contributing to the development of gastroesophageal reflux disease (GERD). [4]

1.3 GASTRIC CANCER CLASSIFICATION

GC is an extremely heterogeneous disease with respect to structure and growth, cell differentiation and molecular pathogenesis. This complexity underlies the diversity of histopathological classification models and the importance of appropriate classification and individualized treatment of GCs. During the last 50 years, the histological classification of GC has been largely based on Lauren's criteria introduced in 1965 [5], in which GC is classified into two major histological subtypes, namely intestinal type and diffuse type adenocarcinoma, in addition to the mixed and indeterminate types. Diffuse carcinomas are poorly differentiated and are constituted of single or poorly cohesive tumour cells, without formation of gland

structure. By contrast, intestinal carcinomas are mostly well differentiated and produce glandular formations reminiscent of colorectal adenocarcinomas, to which the subtype name is due. Diffuse GC is mostly associated with female patients of younger age and presents worse prognosis compared with the intestinal type, which most commonly occurs in elderly male patients and exhibits a better prognosis. [6]

Even if the Lauren model is simple and robust, the more recent World Health Organization (WHO) classification, based on the predominant histological patterns of the carcinoma (tubular, papillary, mucinous, poorly cohesive and rare variants) has the advantage to harmonize with histological criteria of the other gut cancers.

Unfortunately, these traditional morphology-based classification systems have a limited utility in guiding clinical treatment due to the molecular heterogeneity of GC.

Fort his purpose, in 2014 The Cancer Genome Atlas (TCGA) research network has published a molecular classification of GC (Fig.2), identifying four tumour subgroups:

- positive for Epstein-Barr virus (9%), which show frequent *PIK3CA* mutations, high levels of DNA hypermethylation, and amplification of *JAK2, CD274* (also known as *PD-L1*) and *CD273* (also known as *PD-L2*). This molecular subtype is due to infection by the EBV. Early entry of the virus into a single host cell leads to clonal expansion and cancer development.
- microsatellite unstable tumours, (MSI) (22%): this subtype mostly displays the CpG island methylator phenotype (CIMP), characterized by promoter methylation and subsequent transcriptional silencing of several tumor suppressor genes or other tumor-related genes. The main feature is hypermethylation of DNA mismatch repair gene *MLH1*, resulting in a form of genomic instability known as microsatellite instability. Moreover, this subtype presents elevated mutation rates, including mutations of genes encoding targetable oncogenic signalling proteins, such as PD-L1.
- genomically stable tumours (GS) (20%), which exhibit low somatic copy-number aberrations but elevated expression of molecules involved in the cell adhesion and angiogenesis-related pathways, such as recurrent mutations of E-cadherin (*CDH1*), Ras homolog family member A (*RHOA*)

- chromosomally unstable tumours (CIN) (50%), which show marked aneuploidy and amplification of receptor tyrosine kinases-Ras (RTK/RAS) pathway and high frequency of *TP53* mutations (73%), amplification of genes encoding cell cycle mediators.

Although the TCGA group was able to molecularly classify GC in four distinct subtypes, no associated survival difference was observed within its cohort. [7,8]

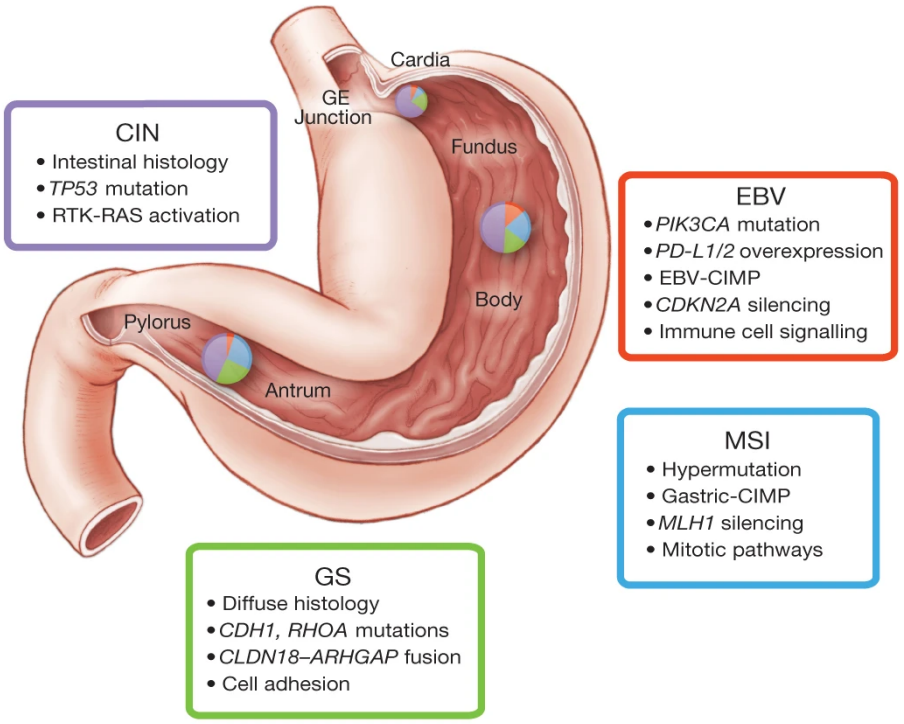

Figure 2. Main features of gastric cancer subtypes. This schematic lists some of the salient features associated with each of the four molecular subtypes of gastric cancer. [7]

In 2015 the Asian Cancer Research Group (ACRG) established clinically relevant molecular classification, partially overlapping that of ATCG, that could overcome the heterogeneity of GC and offer more useful clinical information on the basis of microsatellite status and *TP53* transcriptional activity:

- MSI-high: this subtype had the best prognosis and lowest frequency of recurrence of the four subtypes.
- microsatellite stable/epithelial-mesenchymal transition (MSS/EMT): tumor of this group had a lower number of mutation events but had the worst prognosis and the highest recurrence frequency

- microsatellite stable/epithelial/TP53 intact (MSS/TP53+, p53 active): this subtype had the second best prognosis after MSI subtype.
- microsatellite stable/epithelial/TP53 loss (MSS/TP53-, p53 inactive): this subgroup had the highest rate of *TP53* mutations (60%) with a low frequency of other mutations.

To date, there is not yet an exhaustive classification of GC and, in the future, probably it will be a clinical-pathological-molecular combined stratification to guide individualized approach. [9]

1.4 THE WARBURG EFFECT

One of the most important signatures of cancer is the altered cellular energetics metabolism. Among the several changes of metabolic pathways in tumor cells, a pivotal role is due to increased aerobic glycolysis, which is also known as the Warburg effect. In fact, under aerobic conditions normal cells generally use glucose through oxidative phosphorylation to produce energy. On the contrary, cancer cells mainly produce lactate by means of aerobic glycolysis, even in the presence of sufficient levels of oxygen. Moreover aerobic glycolysis is less efficient in producing energy compared with complete oxidation of glucose, returning only 5% of the energy available from glucose (Fig.3). [10]

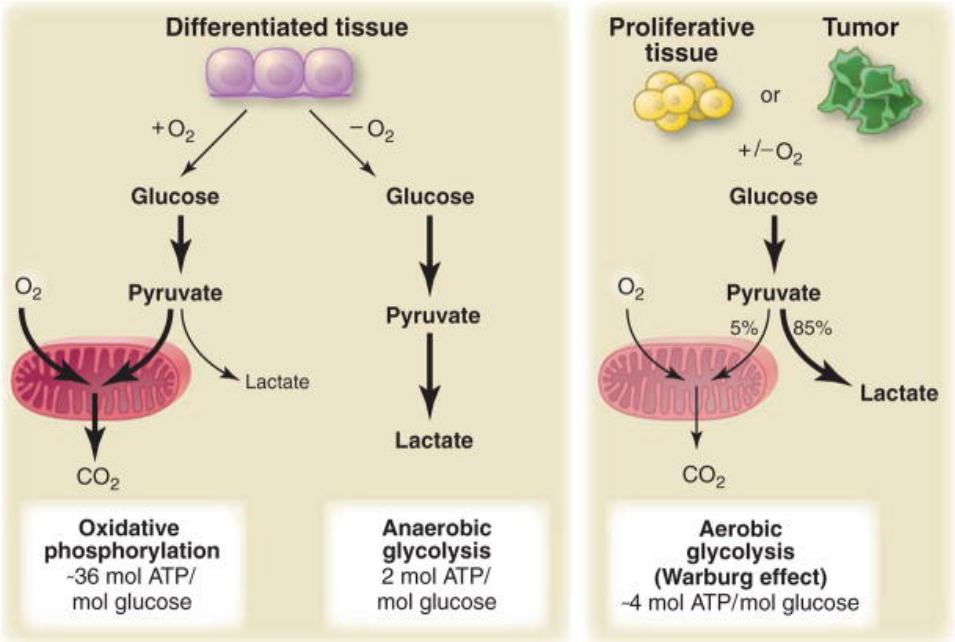

Figure 3. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). [10]

This apparent waste of glucose actually constitutes a survival advantage in rapidly proliferating cells, because it makes them insensitive to transient or permanent hypoxic conditions. Furthermore high levels of lactate causes an acidic microenvironment that has a protective effect on tumor cells and favors tumor invasion by promoting cell migration, angiogenesis, immune escape and radioresistance. [11]

This shift of glucose metabolism is sustained by the upregulation of the key effectors of the glycolytic pathway, including specific membrane transporters of glucose (GLUTs) and all the enzymes that catalyze every single step of the process, and may be significant biomarkers for predicting cancer prognosis and may be therapeutic targets in gastrointestinal cancer (Fig. 4). [11,12]

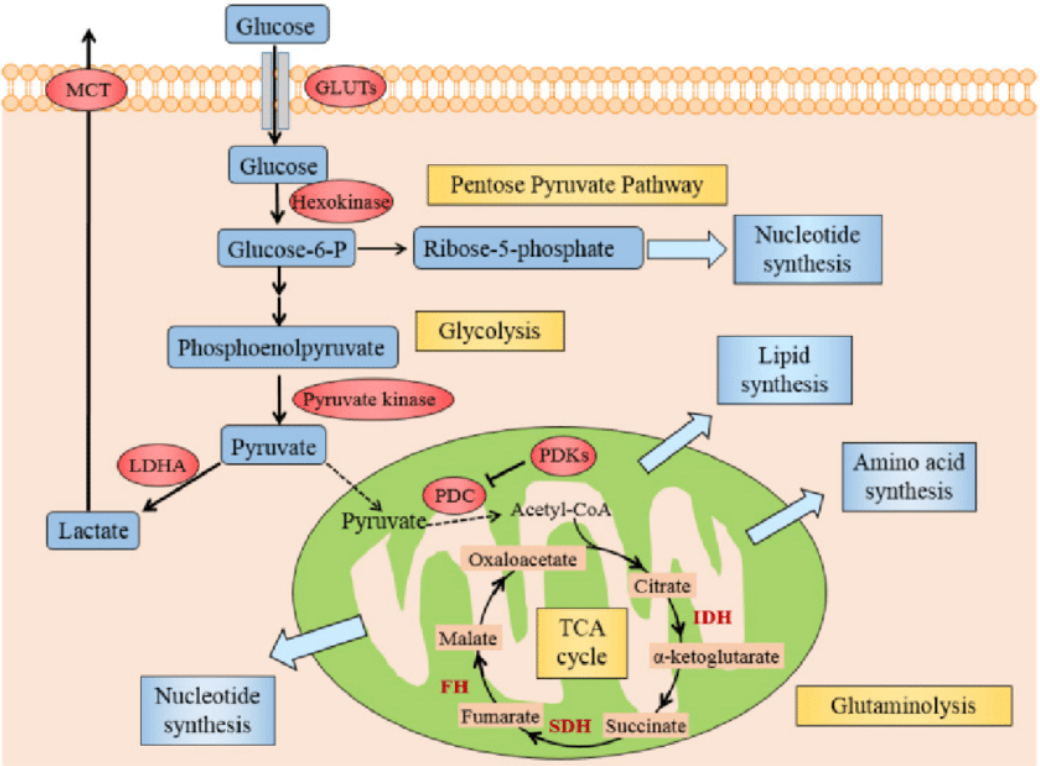

Figure 4. Representation of the impact of Warburg effect in overall energetics metabolism in cancer cells. Major metabolic pathways are shown in yellow boxes and the enzymes controlling key steps in glycolysis are labeled in red. Abbreviations: GLUTs: glucose transporters; MCT: monocarboxylate transporter; PDC: pyruvate dehydrogenase complex; PDKs: pyruvate dehydrogenase kinases; LDHA: lactate dehydrogenase A; HIF1: hypoxia inducible factor 1, IDH: isocitrate dehydrogenase; SDH: succinate dehydrogenase, FH: fumarate hydratase. [W Zhang, Int J Biol Sci. 2015]

Glucose transporters (GLUTs) constitute a family of proteins that regulate the transport of glucose across the hydrophobic cell membranes. Fourteen isoforms of the *GLUT* genes have been identified, which show similar structural architecture but different cellular and subcellular localization, kinetic properties and affinity for glucose and other hexoses. Among the different *GLUT*s, *GLUT1* has been found to be frequently upregulated in a wide variety of cancer types, and its levels of expression are often correlated with the metastatic potential and worse prognosis. [13]

Several other glycolytic enzymes have been confirmed to participate in carcinogenesis and predict the progression of gastric cancer.

Hexokinases (HK) catalyze the phosphorylation of glucose to glucose-6-phosphate, being the first and rate-limiting step in glycolysis. Hexokinase family includes four structurally similar isoenzymes, HK1, HK2, HK3, and glucokinase, but only HK1 and HK2 have similar functions. HK2 localizes to the mitochondria outer membrane and is the most expressed isoform in cancer cells compared with normal tissue. Particularly, it has been demonstrated that high expression of *HK2* is associated with a poor prognosis in gastric cancer. [12]

Pyruvate kinase (PK) controls the final rate-limiting step of glycolysis by catalyzing the dephosphorylation of phosphoenolpyruvate (PEP) to pyruvate and is overexpressed in many human cancers. Four isoenzymes of PK have been reported in mammals: liver-type PK (PKL), red blood cell PK (PKR) and PK muscle isozyme M1 (PKM1) and M2 (PKM2). PKM1 shows high enzymatic activity and is mainly expressed in the muscle and brain, while PKM2 exists in a low-activity form, which becomes prevalent in proliferating cells, both normal and cancer cells. Recent studies have indicated that *PKM2* is overexpressed in gastric cancer and associated with tumor size, invasion and metastasis. [14,15]

Lactate dehydrogenase (LDH) catalyzes the reversible conversion of pyruvate to lactate and exists in five isoenzymes that are similar in function but distinct in tissue distribution. The human isoform LDH-A (or LDH5) is mainly expressed in liver and muscle. Several evidences suggest that *LDH-A*, which is upregulated in invasive cancers, has a critical role in cell proliferation, allowing the survival of tumors even in the presence of low levels of oxygen. [16]

1.5 THE *TP53* GENE IN CANCER

TP53 (Tumor Protein 53) is a multifunctional tumor suppressor gene involved in the control of target genes that regulate many biological processes, including cell-cycle arrest, apoptosis, senescence, energy metabolism and antioxidant defense to prevent tumorigenesis. [17]

Intriguingly, recent studies have disclosed novel functions of the *TP53* including the regulation of glycolysis. [18]

In pre-clinical models, the protein p53 has been shown to repress glycolysis through multiple mechanisms. More in detail, p53 downregulates the expression of glucose transporters and the HK2 and PKM2 glycolytic enzymes. In addition, p53 triggers the expression of *TIGAR* (*TP53*-induced glycolysis and apoptosis regulator), which reduces the intracellular levels of fructose-2,6-bisphosphate, switching glucose catabolism to the pentose phosphate pathway. [19]

In recent years, several evidences also suggested the *TP53* involvement in the control of tumor angiogenesis, probably due to the cross-talk mechanisms between *TP53* and Vascular Endothelial Growth Factor (VEGF) and its receptors. It has been identified a specific p53-binding site within the *VEGF* promoter by which p53 downregulates *VEGF* expression. As a consequence, loss of *TP53* in tumor cells enhances HIF-1alpha levels and augments HIF-1-dependent transcriptional activation of the *VEGF* gene in response to hypoxia. *TP53*-deficient cancer cells were found to produce reactive oxygen species, which activated fibroblasts to mediate angiogenesis by VEGF both *in vivo* and *in vitro*. [20,21] These molecular mechanisms may explain the higher levels of *VEGF* expression frequently found in the presence of *TP53* mutations in cancer tissues and the better efficacy of antiangiogenic treatments in tumors harboring *TP53* mutations. [22]

p53 is one of the most intensively studied tumor suppressor protein because the gene is the most commonly mutated in human cancer. In fact, alterations in p53 expression are required for the development of most cancers, and there is evidence to suggest that restoration or reactivation of p53 function could have significant therapeutic benefit. [23]

Despite decades of research, the analysis of the *TP53* mutational status in cancer therapy for predictive

purposes has not been applied in routine clinical practice yet. Major difficulties regard the absence of standardized methods for determining the *TP53* mutational status in tumor samples. The mutational analysis is more reliable than immunohistochemistry in solid tumors, but somatic *TP53* mutations cannot be considered an homogeneous group inducing an on/off effect. [17]

In fact, *TP53* mutations generally lead to a loss or decreased activity of the protein respect to the wild-type and, as p53 normally acts as a tetramer, these mutant proteins may also function as dominant negative inhibitors over any remaining wild-type p53. [23]

Moreover, it has been observed that some mutant p53 proteins not only lose their tumor-suppressor functions but also may gain novel functions in promoting tumorigenesis as a result of the so-called "gain-of-function" (GOF) *TP53* mutations. [17,23]

The *TP53* is frequently mutated in gastric adenocarcinomas. The reported incidence of p53 mutations in GC ranges from 3.2% to 65%, varying among the different histological types. *TP53* mutation is identified most often in the intestinal type of GC. [24]

Most of the *TP53* mutations are missense and can produce different functional consequences. For example some aminoacid changes may provoke greater dysfunctions or non-functional proteins. To overcome difficulties in the interpretation of the *TP53* mutational analysis for clinical purposes, recently it has been proposed to classify the missense *TP53* mutations by considering the residual transcriptional activity score (RTAS). [25]

Because different studies show that different mutants produce a distinct profile in relation to the loss of "normal" p53 activity, p53 mutants can no longer be considered equivalent. Comprehending the roles of mutant p53 will support the development of new therapeutic strategies for a broad range of cancer types. [23]

1.6 GASTRIC CANCER THERAPEUTIC TREATMENTS

To date, there is no gold standard therapy for GC. Treatment alternatives are usually adopted based on the stage of disease. For early stage disease, tumor resection is the first and preferential option, rather than

systemic chemotherapy, to remove the malignancy. Even if surgery is the unique curative approach in the treatment of GC, the addition of chemotherapy either pre- (neoadjuvant), post- (adjuvant), or peri-operatively has improved the clinical outcome.

In the metastatic setting, first line chemotherapy consists of a platinum-based agent, usually oxaliplatin, and a cytotoxic compound such as 5-Fluorouracil, mainly the FOLFOX or CAPOX. Conversely, for second line therapy Ramucirumab plus Paclitaxel is the preferred regimen. Ramucirumab is a recombinant human immunoglobulin G1 (IgG1) neutralizing monoclonal antibody specific for VEGF receptor-2 (VEGFR2) that prevents ligand binding and receptor-mediated pathway activation in endothelial cells (Fig.5).

It is approved as a single agent, or in combination with paclitaxel, for the treatment of patients with advanced or metastatic gastric or gastroesophageal junction cancer with disease progression. [26]

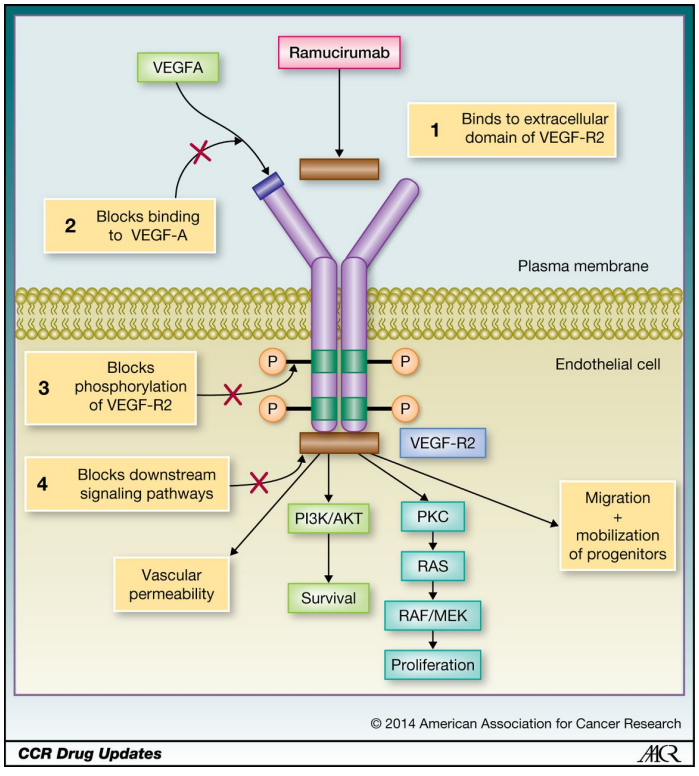

Figure 5. Mechanism of action of Ramucirumab. Ramucirumab binds specifically to VEGFR-2 (part 1), blocks VEGF/VEGFR-2 interaction (part 2), and (part 3) inhibits VEGF-stimulated receptor phosphorylation in endothelial cell resulting in disruption of downstream signaling (part 4). [M Javle, Clin Cancer Res, 2014]

In 2017 FDA also approved Pembrolizumab (PD-L1 monoclonal antibody) for GC with micro-instability high (MSI-H) or mismatch repair deficiency (dMMR) that progressed on previous lines of therapies and do not have other alternative options.

Although there are many therapeutic approaches available for GC patients, most patients die shortly after diagnosis because of (1) the high inter- and intra-tumor heterogeneity and (2) the majority of patients are diagnosed with the metastatic disease.

In the last few years, new-targeted therapies, such as tyrosine kinase inhibitors (TKIs), and immunomodulatory molecules, such as Programmed Cell Death Protein 1 (PD-1) inhibitors, have reached pre-clinical or clinical investigation. [3]

1.7 AIMS OF THE THESIS

Limited data on the altered expression of glucose metabolic effectors in GC *in vivo* are available. Regardless of its origin, this information is relevant because the metabolic shift from oxidative phosphorylation to aerobic glycolysis represents a selective advantage in invasive cancer tissues and it may promote the development of new compounds that target cancer metabolism. The antiangiogenic agent Ramucirumab has become the standard II line therapy for metastatic GC patients but unfortunately clinical response is partial and limited in time. The discovery of predictive biomarkers may facilitate the selection of patients that may benefit from the treatment.

Within this background we planned a first study for evaluating the impact of an altered glycolytic profile in metastatic GC patients treated in II line with Ramucirumab.

Moreover, because mutant p53 can influence the *VEGF* expression, which is the target of the Ramucirumab, a second study was performed to evaluate if the transcriptional activity loss of p53 protein may be predictive in terms of survival outcome in metastatic GC patient treated with Ramucirumab.

CHAPTER 1

GLYCOLYTIC COMPETENCE IN GASTRIC ADENOCARCINOMAS NEGATIVELY IMPACTS SURVIVAL OUTCOMES OF PATIENTS TREATED WITH SALVAGE PACLITAXEL-RAMUCIRUMAB

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Abstract

Introduction: For energy production, cancer cells maintain a high rate of glycolysis instead of oxidative phosphorylation converting glucose into lactic acid. This metabolic shift is useful to survive in unfavorable microenvironments. We investigated whether a positive glycolytic profle (PGP) in gastric adenocarcinomas may be associated with unfavorable outcomes under an anticancer systemic therapy, including the anti-angiogenic ramucirumab.

Materials and methods: Normal mucosa (NM) and primary tumor (PT) of 40 metastatic gastric adenocarcinomas patients who received second-line paclitaxel-ramucirumab (PR) were analyzed for mRNA expression of the following genes: HK-1, HK-2, PKM-2, LDH-A, and GLUT-1. Patients were categorized with PGP when at least a doubling of mRNA expression (PT vs. NM) in all glycolytic core enzymes (HK-1 or HK-2, PKM-2, LDH-A) was observed. PGP was also related to TP53 mutational status.

Results: Mean LDH-A, HK-2, PKM-2 mRNA expression levels were significantly higher in PT compared with NM. 18 patients were classified as PGP, which was associated with significantly worse progression-free and overall survival times. No significant association was observed between PGP and clinical-pathologic features, including TP53 positive mutational status, in 28 samples.

Conclusions: Glycolytic proficiency may negatively affect survival outcomes of metastatic gastric cancer patients treated with PR systemic therapy. TP53 mutational status alone does not seem to explain such a metabolic shift.

Keywords: Glycolysis, Warburg effect, Ramucirumab, Paclitaxel, Angiogenesis

Background

For energy production under aerobic conditions, normal cells generally transform glucose into carbonic anhydride by means of oxidative phosphorylation. Conversely, glycolysis with ultimate production of

lactate is predominant in invasive cancer cells, even in the presence of sufficient levels of oxygen [1]. Although the incomplete oxidation of glucose to lactate yields only 5% of the energy available from glucose, this apparently senseless waste of glucose actually constitutes a survival advantage in rapidly proliferating cells. In fact, it makes them insensitive to transient or permanent hypoxic conditions, it contributes to the production of nucleosides and amino acids, and it constitutes a very rapid way to produce energy [1, 2]. Furthermore, lactate is not just a waste product of this process; on the contrary, it promotes tumor invasion by favouring cell migration, angiogenesis, immune escape and radioresistance [3]. This metabolic shift is promoted by the over-expression of the key effectors of the glycolytic pathway [4, 5], including specific membrane glucose transporters (GLUT-1), and enzymes involved in the promotion of each single step of the glycolytic cascade (Fig. 1).

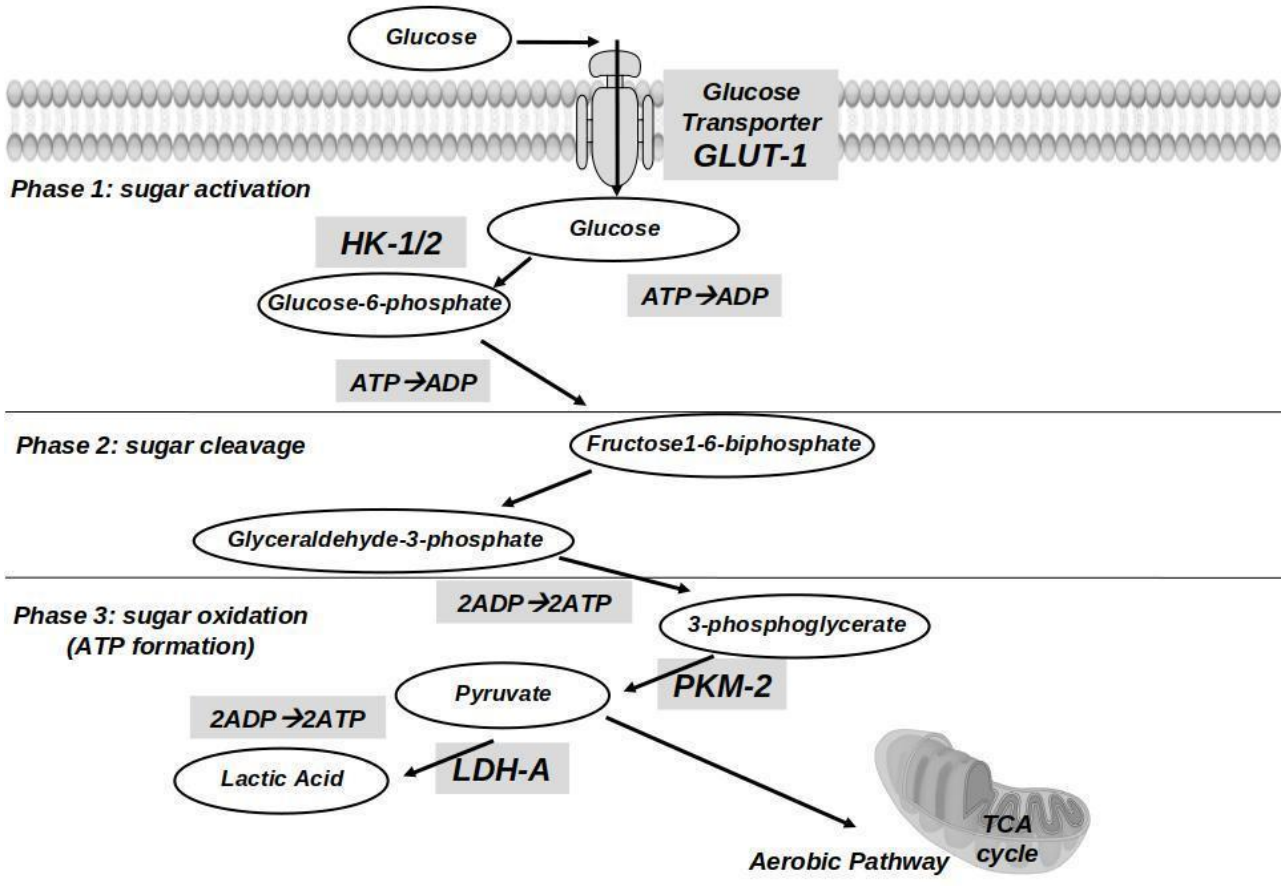

Fig.1 Schematic representation of the three main steps (sugar activation, cleavage and oxidation) in the glycolysis pathway. Glucose transport-1 (GLUT-1) mediates the internalization of glucose across the plasma membrane. Hexokinase (HK-1 and HK-2) transfer one phosphate group from ATP to glucose, yielding glucose-6-phosphate (G6P). G6P may be shunted into the

non-oxidative arm of the pentose phosphate pathway (PPP), otherwise it is converted through the intermediate reaction of glycolysis to 3-phosphoglycerate. Pyruvate kinase (PKM-2) catalyzes the transfer of a phosphate group from 3-phosphoglycerate to ADP, to give pyruvate and ATP. In the presence of oxygen, cells completely oxidize most of that pyruvate in the mitochondria to CO2 during the process of oxidative phosphorylation in the tricarboxylic acid (TCA) cycle. When oxygen is limited, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). Lactate dehydrogenase isoform A (LDH-A) catalyzes the reversible conversion of pyruvate to lactate with the simultaneous oxidation of the cofactor NADH to NAD+. Warburg observed that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis).

The over-expressed enzymes themselves are subject to selection with some isoforms more frequently represented in tumor cells [4, 5]. In a previous study in metastatic colorectal cancer [6], we found mRNA tumor overexpression of GLUT-1 and the glycolytic genes hexokinase 1 (HK-1) and 2 (HK-2), pyruvate kinase isoform 2 (PKM-2) and lactate dehydrogenase isoform A (LDH-A). In the subset of patients treated with anti-angiogenic bevacizumab, the glycolytic profile showed signals of detrimental association with survival outcomes [6]. In fact, clones can be selected that have the ability to survive anti-angiogenic therapy‐induced hypoxia, and the selection of hypoxia ‐resistant clones can also be observed with VEGF (vascular epithelial growth factor) receptor-1 and -2 inhibition [7, 8]. These clones require fewer proangiogenic factors to promote their growth and proliferation and they possess phenotypic properties allowing them to overcome the lack of energy and nutrients supply [7, 8]. Most relevant, metabolic adaptation with a glycolytic shift may not be a simple prognostic biomarker [9, 10], but it may indicate innovative treatment strategies and novel drug targets in anti-cancer therapy [11–13]. This background prompted us to plan a novel study for evaluating the possible negative impact of the up-regulated glycolytic profile in patients exposed to anti-angiogenics. We focused on the paclitaxel-ramucirumab (PR) association for second-line therapy in metastatic gastric cancer. Ramucirumab is a recombinant human immunoglobulin G1 (IgG1) neutralizing monoclonal antibody specific for VEGF receptor-2 that prevents ligand binding and receptor-mediated pathway activation in endothelial cells. It is approved as a single agent, or in combination with paclitaxel, for the treatment of patients with advanced or metastatic gastric or gastroesophageal junction cancer with disease progression or after prior fluoropyrimidine or

platinum-containing chemotherapy [14]. Interestingly, recent studies have revealed novel functions of the TP53 tumor-suppressor gene including the regulation of glycolysis [15]. In pre-clinical models, the p53 protein has been shown to repress glycolysis through multiple mechanisms. In particular, p53 transcriptionally represses the expression of glucose transporters and it was found to down-regulate the HK-2 and PKM-2 glycolytic enzymes. Also, p53 induces the expression of TIGAR (TP53-induced glycolysis and apoptosis regulator), which decreases the intracellular concentrations of fructose-2,6-bisphosphate, and thus reduces glycolysis and diverts glucose catabolism to the pentose phosphate pathway [16, 17]. The TP53 gene is frequently mutated in gastric adenocarcinomas, and unlike many other tumor suppressors, the majority of TP53 mutations are missense, which usually leads to the production of the full-length mutant protein [18]. Also, it has been well documented that some mutant p53 proteins not only lose the tumor-suppressive function, but they gain new oncogenic functions as a result of the so-called "gain-of-function" TP53 mutations [18, 19]. In this study, we also devoted an ancillary analysis to TP53 mutational status to evaluate whether signals of p53 regulation of the glycolytic shift are detectable in vivo.

Methods

Italian institutions involved in the RAMos retrospective study [20] were asked to participate in the present study. To evaluate the results of a translational analysis in a homogeneous population of patients, this retrospective investigation focused on patients treated with the combination of ramucirumab and paclitaxel only. Therefore, patients were required to be treated with ramucirumab 8 mg/kg on days 1 and 15, with paclitaxel 80 mg/m2 on days 1, 8 and 15, and both intravenously every 28 days. Availability of paired tissues of the primary tumor (PT) and normal mucosa (NM) was required for study inclusion. To characterize the glycolytic shift in cancer cells, mRNA over-expression of key enzymes in the three main phases of the glycolytic pathway were studied (Fig. 1). The relationship between levels of the mRNA and survival outcomes was assessed. All patient information and pathology material were collected under a

protocol approved by the Regional Ethical Committee. Patients were asked to provide additional written informed consent (see supplementary information fle).

Samples and nucleic acids extraction

Four to six 10-µm sections from formalin-fixed, paraffin embedded (FFPE) specimens were obtained from PT and matched NM. The sample preparation protocol expressly indicated the acquisition of NM samples from surgical or biopsy blocks with accurate identification of healthy gastric mucosa. These sections had to be distinct from those prepared for tumor sampling thus excluding proximity to tumor infiltration. Before cutting sections for total nucleic acids isolation, an additional slide was prepared for hematoxylin–eosin staining and the pathologists identified representative areas with an almost complete representation of tumor infiltration. For each enrolled patient, total DNA and RNA were extracted from PT and matched NM. Both tissues were micro-dissected and placed in a 1.5 ml reaction tube containing 1 ml xylene to remove paraffin. DNA and RNA were extracted using the RecoverAll™ Multi-Sample RNA/ DNA Isolation Workflow (Invitrogen™, CA, USA) according to the manufacturer's instructions. DNA and RNA concentration and purity were measured using the NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

cDNA synthesis and quantitative real-time PCR (RT-qPCR)

The SuperScriptTMVILO™ cDNA Synthesis kit (Invitrogen™, CA, USA) was used to generate first-strand cDNAs from 1 µg of total RNA according to the manufacturer's instructions. The products were diluted to obtain a final concentration of 10 ng/µl of reverse-transcribed mRNA. Quantitative real-time PCR (RT-qPCR) was performed to analyze the mRNA expression levels of the five candidate genes (HK1, HK-2, PKM-2, LDH-A, and GLUT-1) and two reference genes (B2M and GUSB), as previously reported [6]. Briefy, RT-qPCR was carried out using TaqMan Gene Expression Assay and TaqMan Gene Expression Mastermix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. All reactions were performed in triplicate and each PCR run included a no-template control. The RTqPCR Ct

values (the average value of the triplicates) were converted in Cy0 by a tool for accurate and precise quantification [21] and the relative mRNA expression of each target was calculated as ΔCy0=Cy0(target gene)—Cy0(reference gene). In this analysis, a higher mRNA expression level corresponds to a smaller ΔCy0 value. Subsequently, the 2−ΔΔCy0 method was used to express the n-fold differential expression (fold change) of each candidate gene between the tumor sample and the normal counterpart. Fold change≥2 indicates a doubling in the mRNA content and it was adopted as a threshold for differential RNA expression in microarray and drug induction studies [22–25].

Amplicons library preparation and next-generation sequencing (NGS) for TP53 analysis

A custom panel (IAD_119861) including the TP53 coding and the relative UTR regions was designed using the Ion AmpliSeq™ Designer software (Thermo Fisher, Foster City, CA). The panel was made up of 35 amplicons and ensured 82% of gene coverage. The Ion AmpliSeq Library Kit Plus was used to prepare the libraries according to the manufacturer's instructions. Libraries were generated using 40 ng of tumor DNA and indexed using the Ion Xpress Barcode Adapter Kit. Library purification was carried out using the AMPure™ XP Reagent (Beckman Coulture, CA, USA) on the DynaMag™-2 Magnet. Qubit™ 4 Fluorometer (Invitrogen™,, CA, USA) was used to quantify amplicons libraries. After dilution of all samples at 100 pM, libraries were pooled for emulsion PCR on the Ion OneTouch™ 2 instrument, using the Ion S5™ Template OT2 kit, according to the manufacturer's instructions. The Ion Sphere™ Particles were enriched using the Ion OneTouch™ Enrichment System and the template was sequenced on the Ion Torrent S5 platform using the Ion 540™ Chip following the manufacturer's instruction. All of these instruments and reagents were supplied by Thermo Fisher (Foster City, CA). Read alignment was performed using hg19 (GRCh37) as the reference genome. Variant call files were generated by the Variant Caller v.5 plugin pre-installed in the Torrent Suite and analyzed with the Ion Reporter™ software (Thermo Fisher, Foster City, CA). BAM files were also manually checked on IGV (Integrative Genomics Viewer) [26]. Mutations were categorized as "disruptive" (TP53D) or "non-disruptive" (TP53ND) according to the classification of

Poeta et al. [27]. Mutations were also classified as “gain-of-function” (TP53GOF) if reported in current databases from the review of available studies in which a clear gain-of-function effect was shown [19].

Statistical analysis

mRNA expression levels were reported as ΔCy0 values with means and standard deviations; group differences were compared using two-sample t- and Wilcoxon tests. Significant associations for each gene were required to be detectable with both reference genes. Contingency tables were analyzed by the Fisher’s exact test. Statistical significance was defined as $p<0.05$. For the purpose of clinical associations, fold change results produced by the $2^{-\Delta\Delta Cy0}$ method were adopted. Cases were defined as having a positive glycolytic profle (PGP) when fold changes≥2 (PT vs. NM) were present in all glycolytic core genes: HK-1 or HK-2, PKM-2, LDH-A, and GLUT-1. The remaining cases were categorized as having a negative glycolytic profle (NGP). The primary end-point was overall survival (OS), which was compared between PGP and NGP groups to assess the possible clinical impact of glycolytic proficiency. OS was calculated from the date of the frst cycle of second-line PR therapy to the earliest of date of death or last followup. Progression-free survival (PFS) was the secondary endpoint, defined as the time from the date of the first cycle of second-line PR therapy to the earlier of disease progression or death, or last follow-up. The Kaplan–Meier method was used to estimate survival curves and the log-rank test was used to compare survival times between PGP and NGP groups. A multivariable Cox proportional hazards model was then used to adjust for clinical and pathologic features. Two-sided p values 95% confdence intervals (CI) were reported. A p value<0.05 was considered statistically significant. Statistical analyses were performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium).

Results

Forty consecutive patients who underwent PR second-line systemic therapy and had paired archival tissue samples of the PT and matched NM were included from eight Italian institutions. All patients received frst-line chemotherapy with a platinum derivate (cisplatin or oxaliplatin) plus fluoropyrimidines. In the

second-line setting, patients underwent PR between September 2015 and September 2018 (Table 1). The results of second-line therapy in this cohort of patients parallel findings in the RAMos study [20]. The overall response rate was 17.5%, with 7 partial responses in the 40 patients. In the whole group, the median OS was 7.8 months (95% CI 4.5 to 8.6 months) and the median PFS was 3.8 months (95% CI 3.2 to 4.6 months).

Table 1. Characteristics and distribution of the 40 patients according to the glycolytic profile.

Variable	Number of patients (%)			
	Total	PGP	NGP	p value
Age				
>70 years	29 (72.5)	11 (61)	18 (82)	0.7
≤70 years	11 (27.5)	7 (39)	4 (18)	
Gender				
Male	18 (45)	7 (39)	11 (50)	0.5
Female	22 (55)	11 (61)	11 (50)	
PFS1 time				
>6 months	26 (65)	13 (72)	13 (59)	0.5
≤6 months	14 (35)	5 (28)	9 (41)	
Number of metastatic sites				
1	21 (52.5)	6 (33)	15 (68)	0.05
≥2	19 (47.5)	12 (67)	7 (32)	
Peritoneum involvement				
Positive	21 (52.5)	10 (55)	11 (50)	0.7
Negative	19 (47.5)	8 (45)	11 (50)	
ECOG PS				
0	22 (55)	10 (55)	12 (54.5)	0.5
1-2	18 (45)	8 (45)	10 (45.5)	
Lauren's histology				
Intestinal	26 (65)	14 (78)	12 (54.5)	0.9
Diffuse	14 (45)	4 (22)	10 (45.5)	
Grading				
1-2	18 (45)	10 (55)	8 (36)	0.3
3	22 (55)	8 (45)	14 (64)	
Primary tumor resected				
Yes	18 (45)	7 (39)	11 (50)	0.1
No	22 (55)	11 (61)	11 (50)	
Primary tumor site				
Cardia	9 (22.5)	5 (28)	4 (18)	0.7
non-cardia	21 (77.5)	13 (72)	18 (82)	

Abbreviations: PGP, positive glycolytic profile; NGP, negative glycolytic profile; PFS1, progression-free survival to first-line chemotherapy; ECOG PS, Eastern Cooperative Group Performance Status

Expression analyses

As shown in Fig. 2, statistically significant differences in mRNA expression levels were detected comparing ΔCy0 values between PT tissues and matched NM for HK-2, PKM2, GLUT-1 and LDH-A.

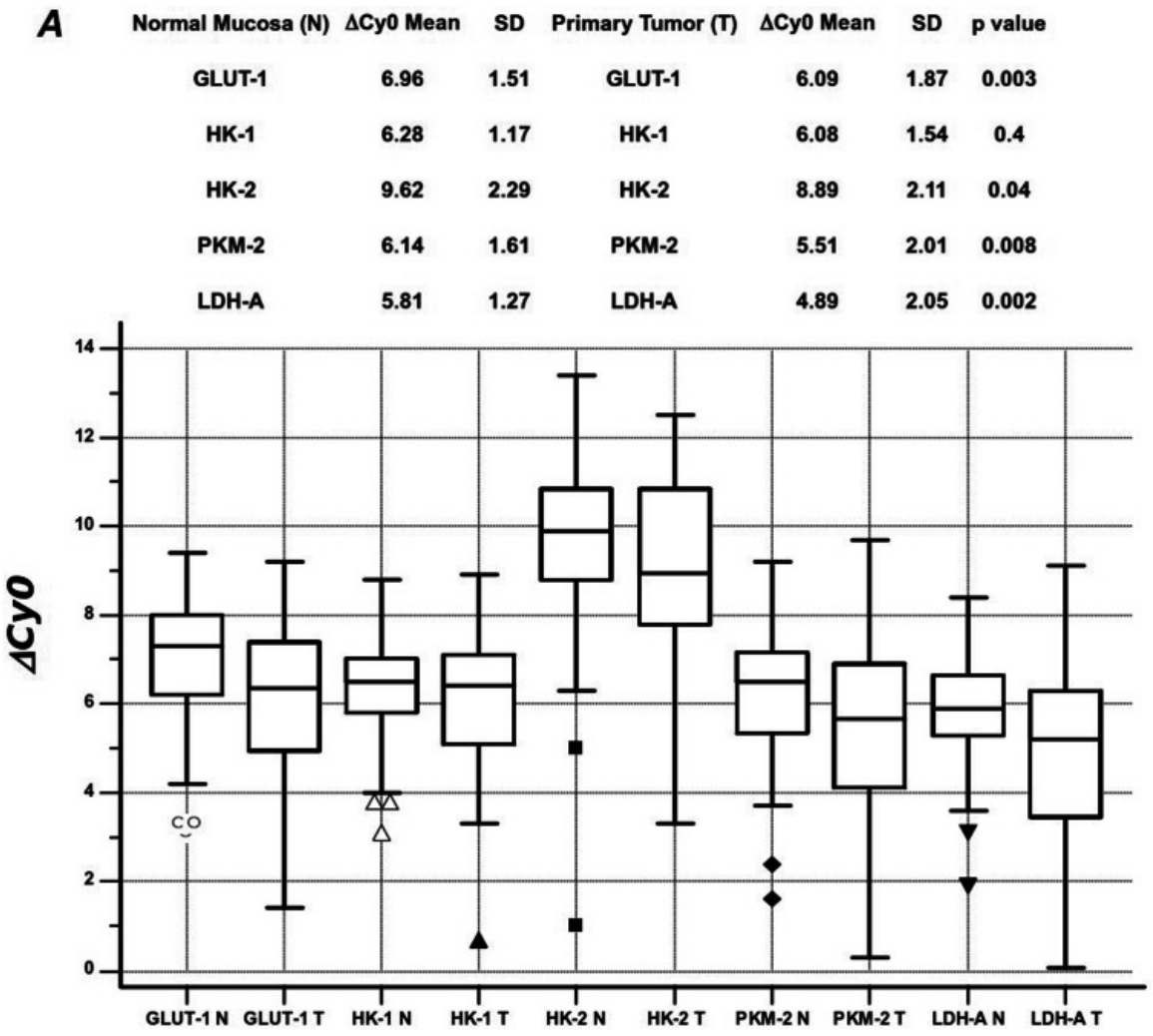

Fig.2 Box plot with standard deviation (SD) bars showing mRNA expression levels of the candidate genes in the primary tumor (T) and normal mucosa (N). Data are presented as ΔCy0 values: the smaller the ΔCy0 value, the higher the expression.

The ranking of fold change analysis is reported in Fig. 3. Fold change≥2 was detected in 19 cases for GLUT-1, in 19 cases for LDH-A, in 19 cases for PKM-2, in 13 cases for HK-2 and in 16 cases for HK-1. Eighteen cases showed synchronous fold change≥2 in one of the two hexokinases (HK-2 or HK-1), PKM-2, LDH-A, and they composed the PGP group. Notably, all the PGP cases showed also fold

change≥2 for GLUT-1. The remaining 22 cases composed the NGP group. Intriguingly, a clear-cut distribution of fold change ≥ 2 expression levels in the four analyzed target genes seems to be present. In fact, except for case number 16 and 30, NGP group cases showed none or a single mRNA fold change≥2.

						TP53 ANALYSIS					
Case number	HK-1	HK-2	PKM-2	LDH-A	GLUT-1	TP53 STATUS	Description	Protein description	Effect	Classification	hg19 Coordinates Chr 17
4						no mutation					
8						no mutation					
13						no mutation					
18						no mutation					
32						point mutation	A>G	Y107X	stop codon	disruptive	7579386
34						no mutation					
36						point mutation	C>T	R196X	stop codon	disruptive	7578263
38						point mutation	C>T	P222L	missense mutation	non-disruptive	7578184
39						point mutation	G>A	R282Q	missense mutation	gain-of-function	7577093
40						point mutation	C>T	R248W	stop codon	disruptive	7577539
2						no mutation					
31						point mutation	C>T; G>A	R342X; E180K	stop codon; missense mutation	disruptive	7574003; 7578392
23						point mutation	C>T; C>T	R181C; R175C	missense mutation	disruptive	7578389; 7578407
24						point mutation	G>A	M246I	missense mutation	gain-of-function	7577543
27						point mutation	C>T	H179Y		gain-of-function	7578395
37						point mutation	C>T	Q317X	stop codon	disruptive	7576896-897
6						no mutation					
1						point mutation	C>T	R282W	missense mutation	gain-of-function	7577094
16						no mutation					
12						no mutation					
15						no mutation					
30						point mutation	G>T	G245C	missense mutation	gain-of-function	7577548
3						point mutation	G>A	R282Q	missense mutation	gain-of-function	7577093
5						point mutation	C>T	P385	missense mutation	non-disruptive	7579395
7						point mutation	G>A	R175H	missense mutation	gain-of-function	7578406
14						no mutation					
20						point mutation	G>A	R282Q	missense mutation	gain-of-function	7577093
21						point mutation	C>T	R282W	missense mutation	gain-of-function	7577094
22						point mutation	C>T; C>T	Q52X; Q16X	stop codon	disruptive	7579533; 7579867
35						point mutation	C>T	R181C	missense mutation	disruptive	7578389
9						point mutation	C>G	P177R	missense mutation	non-disruptive	7578400
10						point mutation	G>A;	G224A		non-disruptive	7578179
11						point mutation	C>T; C>T	A347V; S94L	missense mutation	non-disruptive	7579406; 7573987
17						no mutation					
19						deletion; point mutation	Del; G>A	Intron 8; G226D	remove splice junction; missense mutation	non-disruptive	7576929; 7577604
25						point mutation	C>T; C>G	P130S; P177R	missense mutation	disruptive	7578281; 7578400
26						point mutation	C>T	R306X	stop codon	disruptive	7577022
28						point mutation	C>T	P309S	missense mutation	gain-of-function	7576921
29						point mutation	C>T; G>A	S315F; R283H	missense mutation	non-disruptive	7576902; 7577090
33						point mutation	G>A	R282Q	missense mutation	gain-of-function	7577093

Fig.3 Results of tumor profiling according to fold-change mRNA analysis. Cases were categorized as positive glycolytic profile (PGP) when fold-change ≥ 2 simultaneously occurred in *HK-1* or *HK-2*, *PKM-2*, *LDH-A* (dark grey squares). White squares denote the remaining cases with a negative glycolytic profile (NGP) because fold-change < 2 or fold- change ≥ 2 in only one or two mRNA.

As shown in Table 1, the distribution of clinical and pathologic features of the 40 patients according to PGP and NGP status did not show statistically significant associations. A borderline p value (p=0.05) was observed between PGP and NGP groups for the number of metastatic sites, with a numerically greater prevalence of PGP patients having more extensive metastatic disease. PGP status did not differentiate treatment responses to second-line therapy (p>0.05). There were 2 partial response in the PGP group and 5 in the NGP group. Four patients had stable disease in the PGP group and 10 in the NGP group. Disease progression occurred in 12 patients in the PGP group and in 7 patients in the NGP group. The number of patients with partial response and stable disease in the disease-control rate (DCR) was statistically significantly different between the two groups with DCR in 15 and 6 patients in the NGP and PGP groups, respectively (p=0.03).

Expression analysis and TP53 status

TP53 mutations in the coding region were found in 28 patients (70%). All detected TP53 mutations were missense mutations described in the IARC database. Their characteristics are reported in Fig. 3, together with the distribution of cases according to glycolytic status. Eleven carriers of a TP53 mutation were in the PGP group and 17 were in the NGP group. Seven patients without TP53 mutations were in the PGP group and 5 in the NGP group. The association between TP53 mutation and glycolytic status was not statistically significant (p=0.20). The TP53 missense mutations were classified as TP53ND in 7 cases, TP53D in 10 cases and TP53GOF in 11 cases. In particular, all but one of the TP53ND mutations occurred in the NGP group. Six of the 10 TP53D mutations were in the PGP group and 7 of the 11 TP53GOF mutations were the NGP group (p=0.1).

Survival analysis

In the OS analysis of second-line PR systemic therapy (see Fig. 4, Panel A), patients with a NGP showed statistically significant better survival than those with PGP: median OS of 8.9 months (95% CI 7.8–10.7 months) vs. median OS of 4.1 months (95% CI 3.5–5.3 months), respectively (p=0.008). Similarly,

glycolytic status showed a statistically significant impact on PFS (Fig. 4, panel B). Median PFS in NGP patients was 4.9 months (95% CI 4.4–6.1 months) and median PFS in PGP patients was 2.0 months (95% CI 1.9–3.7 months) (p=0.02).

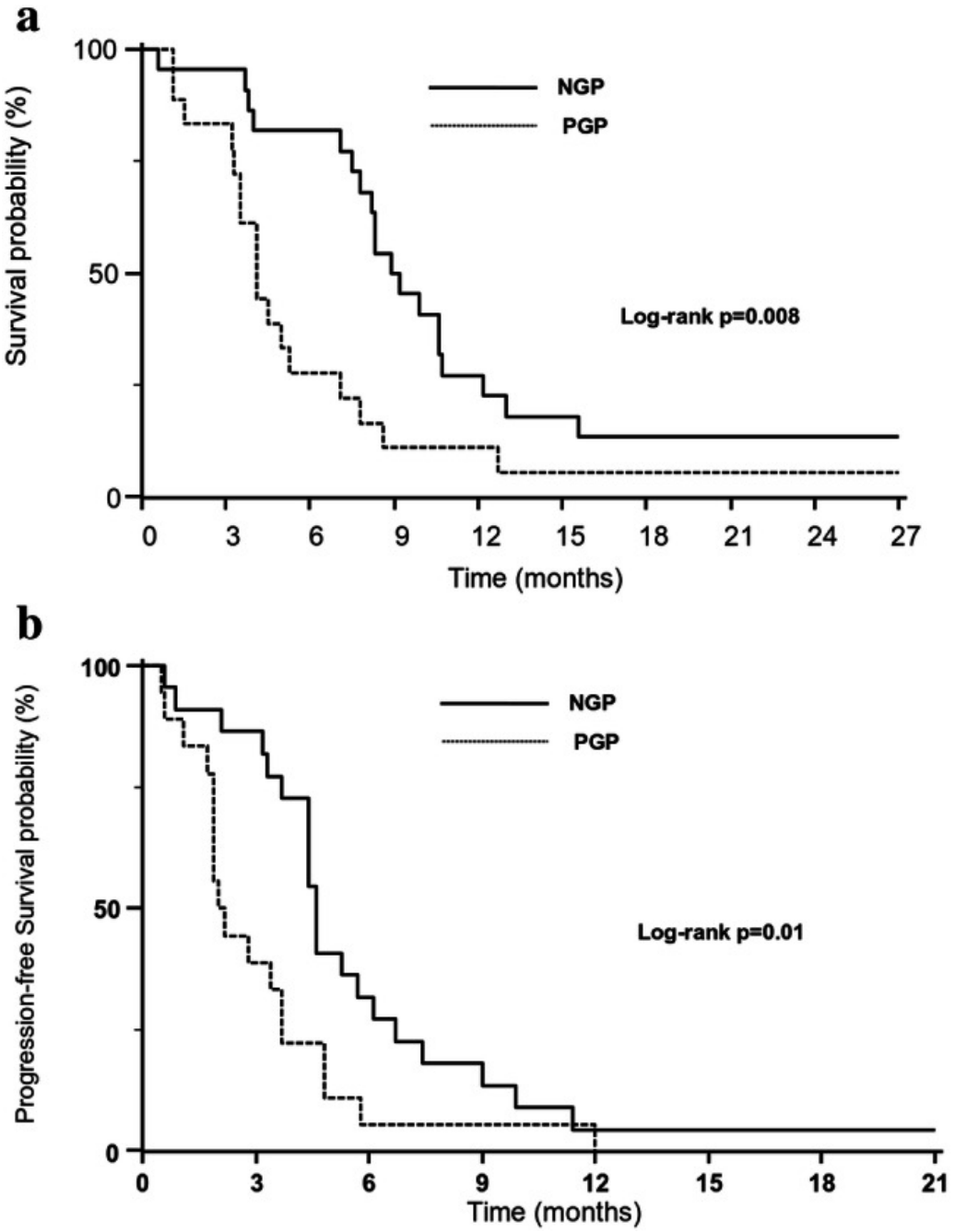

Fig.4 Results of survival analyses by PGP and NGP status in the 40 patients. Kaplan-Meyer survival curves of overall survival (Panel a) and progression-free survival (Panel b) to second-line paclitaxel-ramucirumab.

The results of the multivariable Cox regression analysis are shown in Table 2. The glycolytic profile retained independent associations with PFS and OS, after controlling for other prognostic factors. In the OS analysis, adverse outcome was associated with PGP status, ECOG performance status 1–2, peritoneum involvement and the presence of the primary gastric tumor.

Table 2. Results of the multivariate model analysis

	Overall Survival		**Progression-free Survival**	
Variable	HR (95% confidence interval)	p-value	HR (95% confidence interval)	p-value
Age				
>70 years vs. ≤70 years	0.51 (0.21-1.16)	0.1	0.64 (0.29-1.39)	0.2
Gender				
Female vs, Male	0.53 (0.19-1.46)	0.5	0.48 (0.53-3.78)	0.4
First PFS time				
≤6 months vs, >6 months	2.41 (0.92-6.33)	0.07	1.38 (0.60-3.16)	0.4
Number of metastatic sites				
≥2 vs. 1	1.27 (0.55-2.96)	0.5	1.32 (0.59-2.95)	0.5
Peritoneum involvement				
Positive vs. Negative	2.94 (1.21-7.13)	0.01	1.42(0.60-3.35)	0.4
ECOG PS				
1-2 vs: 0	2.56 (1.18-5.56)	0.01	1.83 (0.88-3.79)	0.1
Lauren's histology				
Intestinal vs. Diffuse	0.46 (0.16-1.33)	0.4	0.64 (0.26-1.54)	0.3
Grading				
3 vs.1-2	1.43 (0.46-4.43)	0.5	1.58 (0.48-5.10)	0.4
Primary tumor resected				
No vs. Yes	3.11 (1.03-9.38)	0.04	3.39 (1.05-10.09)	0.04
Primary tumor site				
Cardia vs. non-cardia	2.55 (0.79-8.26)	0.1	1.81 (0.66-5.39)	0.4
Glycolytic status				
PGP vs. NGP	2.57 (1.17-5.63)	0.01	2.49 (1.16-5.38)	0.01

Abbreviations: HR, Hazard Ratio; PGP, positive glycolytic profile; NGP, negative glycolytic profile; PFS, progression-free survival; ECOG PS, Eastern Cooperative Group Performance Status

Discussion

Even after a decade from the FDA approval of the first anti-VEGF drug bevacizumab, resistance to anti-VEGF therapy remains a challenge in the treatment of cancer patients. Mechanisms of resistance are described as being intrinsic (preexisting) or adaptive (acquired), and they may explain why some tumors do not respond from the outset and why others progress after initial shrinkage. Redundancy of pro-angiogenic growth factors and activation of alternative angiogenic pathways have been investigated and considered as the prevalent mechanisms of resistance to anti-angiogenic compounds in cancer therapy [8, 9]. In recent years, many pre-clinical and translational studies indicated that metabolic reprogramming with adaptive escape in response to a hypoxic tumor microenvironment may offer a novel and intriguing opportunity for explaining the failure of anti-angiogenic treatments in solid tumors. In particular, glycolysis is an essential metabolic pathway in the hypoxic adaptation for survival and tumor progression. In this perspective, tumors may develop early and/ or late resistance to anti-angiogenic agents when clones are more equipped for prompt and redundant metabolic changes (i.e., glycolytic shift) in a therapeutically induced hypoxic environment [7, 9]. In the past few years, translational clinical studies in cancer patients have addressed the putative clinical impact of glycolysis-related proteins and factors on prognosis, and there is mounting evidence that these features negatively affect survival outcomes [28]. The gastric cancer setting was analyzed in some of these studies. Findings showed significant associations between poor prognosis and more advanced stage disease or adverse histological features with up-regulated expression of glucose transporters [29–31] hexokinases [32–34] pyruvate kinases [34, 35], other enzymes involved in energy metabolism [36, 37], and lactate dehydrogenase [38–40]. To the best of our knowledge, this is the first study that analyzes a homogenous population of metastatic gastric cancer patients treated with a regimen that includes an anti-angiogenic compound. Moreover, we attempted an approach with multiple genes to determine a PGP rather than a single-component analysis. A starting point for discussion is the characterization of the population of gastric cancer patients whose tumors displayed the positive glycolytic profile. Given the relatively early interest in the clinical impact of cancer metabolic features, there is a lack of standardized criteria and almost all studies investigated single glycolysis-related factors with different

methods [28]. We formulated an approach which combined the biochemical principle of the glycolytic cascade in its main enzymatic steps [41], together with a sensitive threshold for mRNA expression in vivo [23, 25]. Fold-change is a very intuitive method to identify differentially expressed genes and it quantifies the change of expression levels. In fold-change analyses, 1.5 or 2 is often used as the cut-of to choose differentially expressed genes. The contemporary up-regulation of three key glycolytic enzymes coupled with fold-change ≥ 2 in mRNA expression in cancer tissues compared with their normal counterpart is a fairly strict criterion to label a PGP case. In a comparative analysis between tissues, the quality of sampling is mandatory for reducing the risk of biases. For example, the result of a NGP status could be a false negative if sampling was made in deceptively healthy NM areas. In our study, pre-specified procedures for sampling and the involvement of expert pathologists in the selection of tissues should have minimized this risk. The observed differences in mRNA expression levels of target genes between NM and PT tissues would suggest an effective procedure to seize the presence of the glycolytic shift. We cannot rule out that an expression level analysis of each target gene in PT or NM tissues could be also predictive of clinical outcomes. However, to our opinion, this approach would be less informative on a global glycolytic shift and it could be done after identifying a cut-of level for each tested gene. We acknowledge that our criteria necessitate replication in independent studies and additional settings. Hopefully, they could lay the groundwork for a standardized determination of a glycolytic profile in translational cancer studies In the global analysis of mRNA levels between PT tissues and NM, enhanced expression of GLUT-1, PKM-2, LDH-A and HK-2 was found. Except for HK-1, these data parallel our findings in colorectal cancer [6]. The variable result of HK-1 expression analysis in cancer tissues and the lack of global up-regulation in this study is not surprising. HK-2 was found to be overexpressed more than HK-1 in several cancer types compared with their normal counterpart [42]. The four hexokinase isoenzymes (HK-1, HK-2, HK-3, and glucokinase) are structurally similar, but only HK-1 and HK-2 are functionally similar. Since hexokinase serves as the gateway through which glucose enters the alternative metabolic pathway, HK-1 is redundant to the primary catalytic role of HK-2 to ensure the cancer cell a constant glycolytic flux [43]. According to our criteria, 18 (45%) primary gastric adenocarcinomas were categorized as having a positive glycolytic

profile and, therefore, displaying an intrinsic capability of metabolic adaptation in an unfavorable, hypoxic microenvironment. In fact, the PR combination is not a "pure" antiangiogenic treatment, but it has been demonstrated that even paclitaxel exploits anti-angiogenic mechanisms of action, especially in fractionated regimens [44]. These features may explain why the exposure of these patients to an anti-angiogenic therapy with ramucirumab and paclitaxel produced significantly shorter OS and PFS than patients with a negative glycolytic profile. More than providing novel prognostic information, mediators of an up-regulated glycolytic status may represent the target of novel compounds with tumor metabolism interference activity [45]. Inhibitors of glucose transporters, PKM-2 and LDH-A, attenuate aerobic glycolysis and tumor proliferation with the potential therapeutic role [46], alone or in combination with anti-angiogenic [47] and immune checkpoint [48] therapies. Unlike our previous analysis in colorectal cancer [6], we did not evaluate RAS mutations. This choice was made considering the low frequency of RAS mutations in gastric cancer (roughly 1–10%) and the presence instead of RAS amplification [49]. Notably, RAS mutations and RAS amplification may display different oncogenic effects in molecular pathways [49], thus, making it even more difficult to interpret the role of oncogenic RAS in gastric adenocarcinoma. Conversely, we attempted an exploration between glycolytic status with PGP/NGP categories and TP53 mutations. This analysis was supported by high frequency of TP53 mutations in gastric adenocarcinomas [18, 19] and the increasing amount of pre-clinical and experimental data, which support a major role of the tumor suppressor gene in orchestrating the glycolytic capability of cancer cells [15, 17]. TP53 mutations were almost equally distributed between PGP and NGP, without any significant association even after considering their sub-classification into disruptive, non-disruptive and gain-of-function.Proof of TP53 driving the Warburg effect mainly derive from pre-clinical studies in cellular and murine models [15, 17]. It is likely that glycolytic properties of cancer cells in vivo undergo modulation from multiple effectors [50]. Therefore, the putative influence of p53 perturbation is diluted among the several factors that may impact the glycolysis capabilities of cancer cells. Limitations of this study are its retrospective nature and small sample size. However, our sophisticated analysis of glycolysis profiling in gastric adenocarcinomas, which required primary tumor tissues and the normal mucosa, lays a foundation for future study. This is especially

important since no affordable predictive marker of response and survival under the ramucirumab-paclitaxel regimen has been identified [51] and second-line therapy is offered to all patients who may benefit according to clinical criteria. In conclusion, glycolytic proficiency was found to be associated with adverse survival outcomes of metastatic gastric cancer patients treated with PR systemic therapy. TP53 mutational status alone does not seem to explain such a metabolic shift. Further investigation is needed to confirm our findings that would promote the development of novel therapeutic strategies against cancer metabolism.

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CHAPTER 2

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TP53 MUTATION ANALYSIS IN GASTRIC CANCER AND CLINICAL OUTCOMES OF PATIENTS WITH METASTATIC DISEASE TREATED WITH RAMUCIRUMAB/PACLITAXEL OR STANDARD CHEMOTHERAPY

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Abstract

Loss of p53 promotes vascular endothelial growth factor (VEGF)-A up-regulation and the angiogenic potential of cancer cells. We investigated TP53 somatic mutations in 110 primary gastric adenocarcinomas of two retrospective metastatic series including 48 patients treated with second-line Ramucirumab/Paclitaxel and 62 patients who received first-line chemotherapy with Cisplatin or Oxaliplatin plus 5-Fluorouracil. Missense mutations were classified by tumor protein p53 (TP53) mutant-specific residual transcriptional activity scores (TP53RTAS) and used to stratify patients into two groups: transcriptionally $TP53_{Active}$ and $TP53_{Inactive}$. The primary endpoint was overall survival (OS). An additional analysis was addressed to measure VEGF/VEGF receptor 2 (VEGFR2) expression levels in relation to the TP53RTAS. In the Ramucirumab/Paclitaxel group, 29/48 (60.4%) patients had TP53 mutations. Ten patients with $TP53_{Inactive}$ mutations showed better OS than carriers of other TP53 mutations. This effect was retained in the multivariate model analysis (Hazard Ratio = 0.29, 95% confidence interval = 0.17–0.85, p = 0.02). In the chemotherapy group, 41/62 (66%) patients had TP53 mutations, and the 11 carriers of $TP53_{Inactive}$ mutations showed the worst OS (Hazard Ratio = 2.64, 95% confidence interval = 1.17–5.95, p = 0.02). VEGF-A mRNA expression levels were significantly increased in $TP53_{Inactive}$ cases. Further studies are warranted to explore the effect of $TP53_{Inactive}$ mutations in different anti-cancer regimens. This information would lead to new tailored therapy strategies for this lethal disease.

Keywords: gastric cancer; TP53; Ramucirumab; Paclitaxel; angiogenesis

Introduction

Tumor protein p53 (TP53) is a multifunctional tumor suppressor gene that is intimately involved in the control of target genes that regulate “healthy” biological processes, including cell-cycle arrest, apoptosis,

senescence, energy metabolism, and antioxidant defense to prevent tumorigenesis [1]. In recent years, several experimental and clinical studies have also indicated a role for TP53 in the control of tumor angiogenesis [2]. This effect seems to be linked to cross-talk mechanisms between TP53, vascular endothelial growth factor (VEGF), and VEGF receptors. A highly conserved and functional p53-binding site has been identified within the VEGF promoter and the p53 tumor suppressor downregulates VEGF expression [3]. Loss of TP53 in tumor cells enhances HIF-1alpha levels and augments HIF-1-dependent transcriptional activation of the VEGF gene in response to hypoxia [4]. TP53-deficient cancer cells were found to produce reactive oxygen species, which activated fibroblasts to mediate angiogenesis by VEGF both in-vivo and in-vitro [5]. The transcription factor E2F1 showed regulation of angiogenic activity via p53-dependent transcriptional control of VEGF expression [6]. In experimental models, mutant TP53 can up-regulate the transcription of VEGF receptor 2 (VEGFR2) by promoter remodeling [7]. These molecular mechanisms may explain analyses of human cancer tissues that have reported significant increases in VEGF expression levels in the presence of TP53 mutations [8–10]. Interestingly, in a large pan-cancer study [9], the association between VEGF up-regulation and TP53 mutants remained independent of HIF-1 and MDM2 overexpression. This translational background explains recent clinical findings in advanced cancer patients who had improved responses and survival outcomes after VEGF/VEGF receptor (VEGFR) inhibitor therapy mostly in tumors harboring a TP53 mutation [11–15]. The concept that TP53 alterations may represent a favorable biomarker for treating patients with anti-angiogenesis agents contrasts with previous findings from standard chemotherapy studies, where TP53 dysregulation was generally associated with poor clinical outcomes [16]. However, this is not surprising considering the multiple and widespread roles of TP53 and the prevalence of p53-associated mechanisms of chemoresistance [16]. Despite decades of research, the analysis of the TP53 status for predictive purposes in cancer therapy has not been implemented in routine clinical practice yet. Major limitations concern the lack of standardized methods for defining the TP53 status in tumor samples. Mutational analysis is more reliable than immunohistochemistry in solid tumors, but somatic TP53 mutations cannot be considered a homogeneous group inducing an on/off effect [1]. The majority of TP53 mutations occurring in human solid neoplasms are missense mutations

with a large gradient of functional consequences [1]. Missense TP53 mutations can be classified for clinical purposes by considering the residual transcriptional activity score ($TP53_{RTAS}$) [17], derived from the results of a site-directed mutagenesis technique and yeast-based functional assay [18]. Gastric cancer ranks among the most frequently TP53-mutated solid tumors [19], and in recent years, the anti-VEGFR2 inhibitor Ramucirumab coupled with Paclitaxel has become standard second-line systemic therapy in this lethal disease [20]. Unfortunately, the magnitude and the duration of the survival gain in Ramucirumab/Paclitaxel treated patients are limited and the discovery of predictive markers would improve the selection of patients and allow the adoption of novel combination therapies [21]. This background prompted us to plan a translational study in patients with metastatic gastric cancer treated with Ramucirumab/Paclitaxel including the analysis of TP53 mutations and $TP53_{RTAS}$ in their tumor samples. The association between the mutant TP53 functional status and survival outcome was assessed and overall patient survival was the primary endpoint of the study. To better characterize the predictive impact of TP53 mutations, an additional retrospective cohort of patients treated with standard chemotherapy for advanced disease was included in the study.

RESULTS

The overall study population consisted of 110 gastric cancer patients whose primary tumors were analyzed for TP53 mutations. The study group included 48 cases who underwent second-line Ramucirumab/Paclitaxel. In the control group, 62 patients were treated with standard first-line chemotherapy with a 5-Fluorouracil and a platinum compound (Cisplatin or Oxaliplatin).

TP53 Analysis in Primary Gastric Tumors

As shown in Table 1, 61 TP53 mutations were detected in total, including 47 missense mutations (77%), 7 nonsense mutations (11.4%), 4 frameshift mutations (6.6%), 2 splice site mutations (3.3%), and 1 in-frame deletion (1.7%). Some “hot-spot” missense mutations occurred in more than one patient: p.R282W and p.G244D in two cases, p.R283H in three cases, p.R273C in five cases. Four patients showed a combination

of two or more TP53 mutations in their tumor samples. Overall, 70 out of 110 patients showed tumor samples positive for TP53 mutations (63.6%). The distribution of TP53 mutations (any type) according to clinical and pathological characteristics of patients and tumors is shown in Table 2. No significant association was found except for a prevalence of TP53 mutations in intestinal-type gastric cancer according to Lauren's classification (Table 2).

Table 1. Description of the tumor protein p53 (*TP53*) mutations detected in 70 patients.

Mutation	Amino Acid Change	Effect	RTAS	Functional Classification	Hg19 Coordinates	Therapy Group
G > T	G245C	missense mutation	0	Inactive	7577548	R/P-SC
G > A	M246I	missense mutation	0	Inactive	7577543	R/P
C > T	R248W	missense mutation	0	Inactive	7577539	R/P-SC
C > T	R282W	missense mutation	0	Inactive	7577094	R/P [2]-SC
G > A	R283H	missense mutation	0	Inactive	7577090	R/P [3]
C > T	T304I	missense mutation	0	Inactive	7577027	R/P-SC
G > A	G244D	missense mutation	0.2	Inactive	7577550	R/P-SC [2]
C > T	R273C	missense mutation	0.4	Inactive	7577121	SC3 [5]
G > A	V216M	missense mutation	1.2	Active	7578203	SC
C > T	P151S	missense mutation	5.2	Active	7578479	SC
G > A	R175H	missense mutation	9.2	Active	7578406	R/P-SC
T > C	I195T	missense mutation	11.4	Active	7578265	SC
C > G	P177R	missense mutation	12.0	Active	7578400	R/P
C > T	L194F	missense mutation	12.0	Active	7578269	SC
C > T	S260F	missense mutation	12.6	Active	7577502	SC
G > A	G105S	missense mutation	15.0	Active	7579374	SC
C > T	H214Y	missense mutation	20.9	Active	7578209	SC

Mutation	Amino Acid Change	Effect	RTAS	Functional Classification	Hg19 Coordinates	Therapy Group
C > T	H179Y	missense mutation	22	Active	7578395	R/P
G > A	E180K	missense mutation	22.8	Active	7578392	R/P
C > T	P177S	missense mutation	26.9	Active	7578401	SC
G > A	R282Q	missense mutation	30.5	Active	7577093	R/P
C > T	P190S	missense mutation	32.0	Active	7578281	SC
C > T	R181C	missense mutation	32.4	Active	7578389	R/P
G > A	D228N	missense mutation	40.7	Active	7577599	SC
G > A	C229Y	missense mutation	69.3	Active	7577595	SC
C > T	R175C	missense mutation	72.5	Active	7578407	R/P
C > T	L252F	missense mutation	76.7	Active	7577527	SC
G > A	R379H	missense mutation	77.8	Active	7572974	SC
C > T	H115Y	missense mutation	81.1	Active	7679344	R/P
G > A	G356R	missense mutation	88.3	Active	7573961	SC
C > T	S116F	missense mutation	90.7	Active	7579340	SC
G > A	V225I	missense mutation	91.7	Active	7577608	R/P
G > A	A353T	missense mutation	96.9	Active	7573970	SC
C > T	L383F	missense mutation	97.5	Active	7572962	R/P
C > T	S90F	missense mutation	99.2	Active	7579418	SC
G > A	R174K	missense mutation	102.0	Active	7578409	SC
C > T	P222L	missense mutation	102.9	Active	7578184	R/P
G > A	E294K	missense mutation	107.7	Active	7577058	SC
G > A	S261N	missense mutation	108.0	Active	7577499	SC
C > T	S314F	missense mutation	110.0	Active	7576905	SC
G > A	V217M	missense mutation	116.0	Active	7578200	SC

GCCCCCTCC > gCCCCTCcc	APS88-90VPS	reading frameshift	-	Other	7579419-424	R/P
AGA > A	R209X	reading frameshift	-	Other	7578221-223	SC
CCT > -	P190-	inframe deletion	-	Other	75782780-281	R/P
G > T	-	acceptor intron 8	-	Other	7576927	R/P
G > A	-	acceptor intron 9	-	Other	7576852	SC

Abbreviations: RTAS, residual transcriptional activity score; SC, standard chemotherapy; R/P, Ramucirumab/Paclitaxel; hg19, Genome Reference Consortium Human Build 37 (GRCh37) coordinates; Legend: [2] mutation in two cases; [3] mutation in three cases; [5] mutation in five cases; * stop codon.

Table 2. Characteristics and distribution of the 110 patients according to treatments and *TP53* status.

	Number of Patients (%)						
Variable	**Ramucirumab/Paclitaxel**		**Standard Chemotherapy**		**Total**		***p*-Value**
	TP53 wt	TP53 mut	TP53 wt	TP53 mut	TP53 wt	TP53 mut	
Age							
>65 years	12 (63.2)	15 (51.7)	11 (52.4)	20 (48.8)	23 (57.5)	35 (50)	0.5
≤65 years	7 (36.8)	14 (48.3)	10 (47.6)	21 (51.2)	17 (42.5)	35 (50)	
Gender							
Male	11 (57.9)	16 (55.2)	15 (71.4)	23 (56.1)	26 (65)	39 (55.7)	0.4
Female	8 (42.1)	13 (44.8)	6 (28.6)	18 (43.9)	14 (35)	31 (44.3)	
Grading							
1–2	16 (84.2)	20 (68.9)	14 (66.6)	24 (58.5)	30 (75)	44 (62.8)	0.2
3	3 (15.8)	9 (31.1)	7 (33.4)	17 (41.5)	10 (25)	26 (37.2)	
Peritoneum involvement							
Positive	10 (52.6)	16 (55.2)	3 (14.3)	14 (34.2)	13 (42.5)	30 (42.8)	0.4
Negative	9 (47.4)	13 (44.8)	18 (85.7)	27 (65.8)	27 (67.5)	40 (57.2)	
ECOG PS							
0	9 (47.3)	18 (62.1)	18 (85.7)	24 (58.5)	27 (67.5)	42 (60)	0.5
1–2	10 (52.7)	11 (37.9)	3 (14.3)	17 (41.5)	13 (42.5)	28 (40)	
Lauren's histology							
Intestinal	12 (63.2)	23 (79.3)	11 (52.4)	32 (78)	23 (57.6)	55 (78.5)	0.02
Diffuse	7 (36.8)	6 (20.7)	10 (47.6)	9 (22)	17 (42.5)	15 (21.5)	
Grading							
1–2	10 (52.6)	19 (65.5)	12 (57.1)	31 (75.6)	22 (55)	50 (71.4)	0.09
3	9 (47.4)	10 (34.5)	9 (42.9)	10 (24.4)	18 (45)	20 (28.6)	
Primary tumor resected							
Yes	12 (63.2)	9 (31.1)	10 (52.4)	18 (43.9)	22 (55)	27 (38.5)	0.1
No	7 (36.8)	20 (68.9)	11 (47.6)	23 (56.1)	18 (45)	43 (61.5)	
Primary tumor site							
Cardia	7 (36.8)	11 (37.9)	9 (42.9)	15 (36.5)	16 (40)	26 (37.1)	0.8
non-cardia	12 (63.2)	18 (62.1)	12 (57.1)	26 (63.5)	24 (60)	44 (62.9)	

Abbreviations: wt, wild-type; mut, mutated; ECOG PS, Eastern Cooperative Group Performance Status.

Classification of TP53 Mutations and Study Groups

Results of the residual transcriptional activity score (RTAS) analysis for missense mutations ($TP53_{RTAS}$) are listed in Table 1. $TP53_{Inactive}$ missense mutations were found in 10 patients in the Ramucirumab/Paclitaxel group and 11 patients in the chemotherapy control group. The remaining 49 TP53 mutation-positive patients were classified as carriers of a $TP53_{Active}$ missense mutation and carriers of non-missense mutations

(nonsense, frameshift, splice-site, and in-frame deletions). $TP53_{Active}$ missense mutation carriers were in 13 cases in the Ramucirumab/Paclitaxel group and 25 cases in the chemotherapy control group. Non-missense mutations carriers totaled 5 in the Ramucirumab/Paclitaxel group and 6 in the chemotherapy control group.

Ramucirumab/Paclitaxel Second-Line Therapy and TP53 Analysis

In the 48 patients of the study group, the results of the second-line therapy showed a 20.8% overall response rate (10 patients with a partial response) and a median overall survival (OS) time of 8.4 months (5–8.8 months 95% CI). No significant association was detected between TP53 mutations and tumor response. Partial responses occurred in three patients with $TP53_{Inactive}$ missense mutations, in two patients with TP53 non-missense mutations, and in five patients with wild-type $TP53_{RTAS}$ status. Median OS times were: 9.5 months (9.0–10.7 months 95% CI) in carriers of TP53Inactive missense mutations; 8.6 months (5.9–9.9 months 95% CI) in carriers of other TP53 mutations; 6.0 months (3.2–8.5 months 95% CI) in carriers of TP53Active missense mutations; 4.5 months (4.1–8.2 months 95% CI) in patients without TP53 mutations. A significant difference was observed between the survival curves of the four groups using the log–rank test (Figure 1). The analysis of hazard ratios with 95% CIs indicates the survival advantage of carriers of TP53Inactive missense mutations over other groups except for carriers of other TP53 mutations (Figure 1). The favorable effect of the $TP53_{Inactive}$ mutational status was retained in the multivariate model (Figure 2).

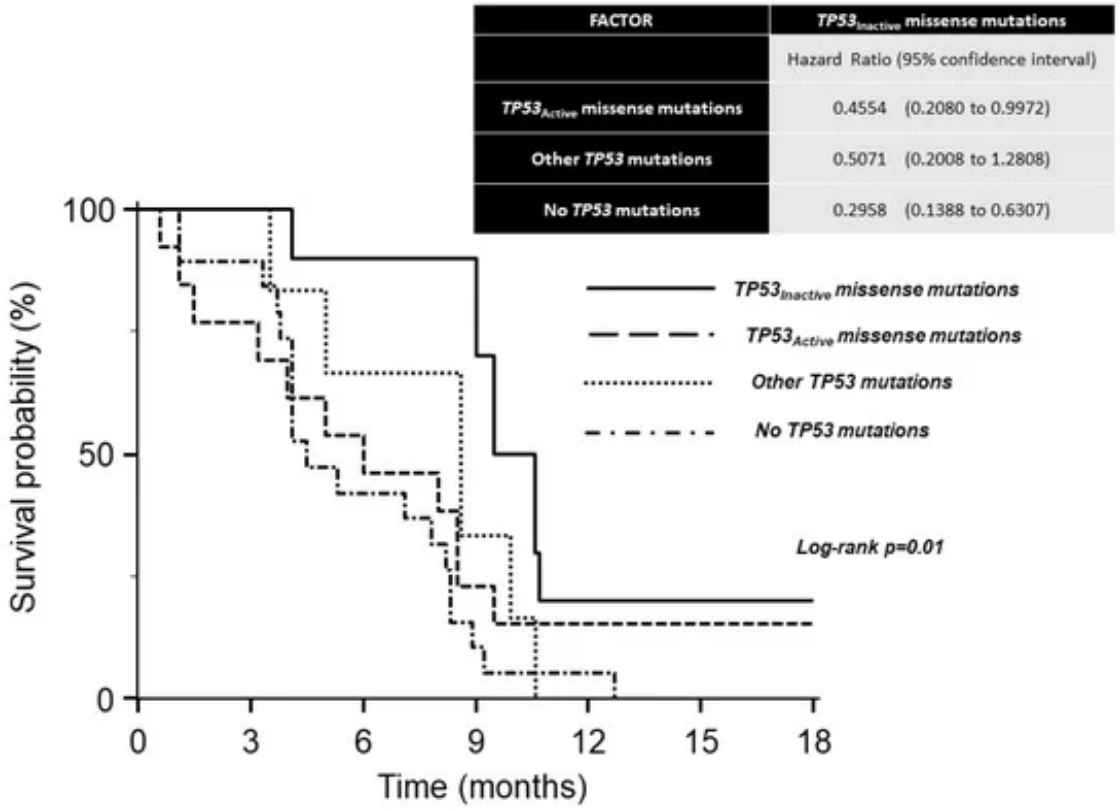

Fig.1 Kaplan-Meier survival curves of Ramucirumab/Paclitaxel second-line therapy in 48 patients with metastatic gastric cancer.

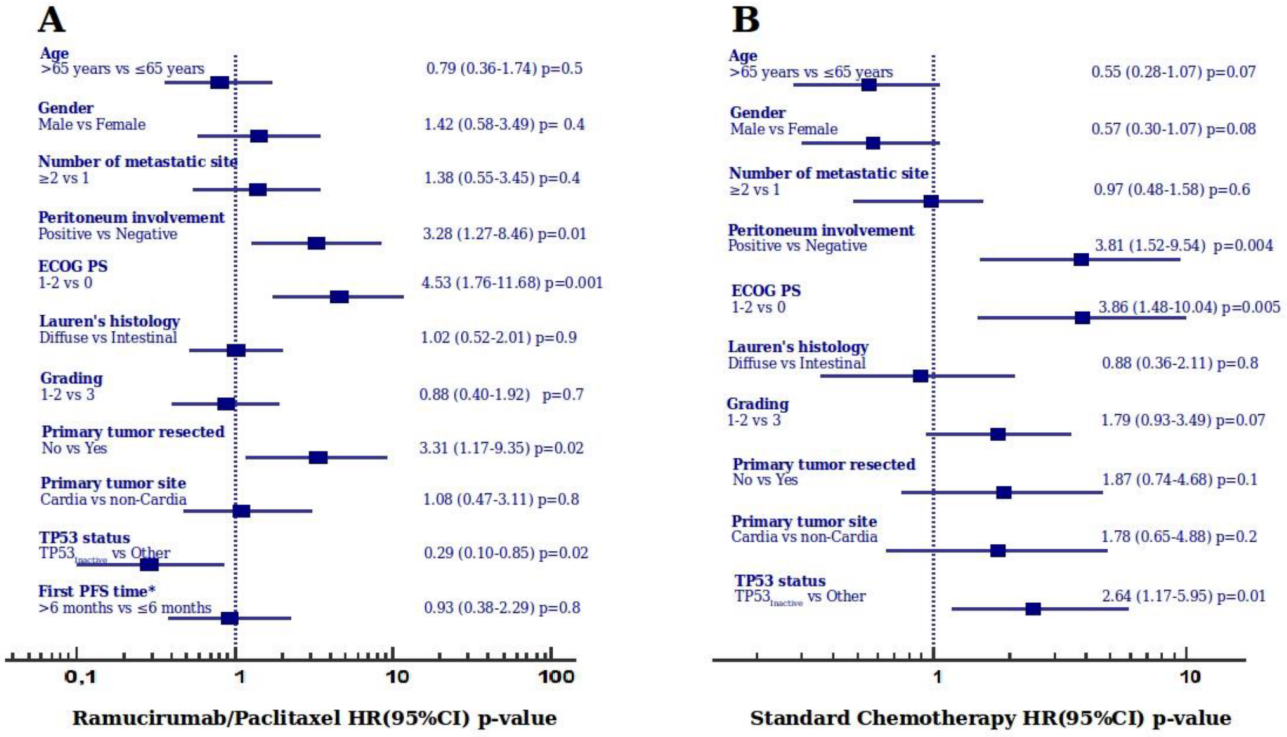

Fig.2 Results of the multivariate model analysis for overall survival in the Ramucirumab/Paclitaxel (A) and Standard Chemotherapy (B) treatment groups. Abbreviations: HR, Hazard Ratio; CI, Confidence Interval; ECOG PS, Eastern Cooperative

Group Performance Status; PFS, Progression-Free Survival. First PFS time* is available for second-line Ramucirumab/Paclitaxel only.

Standard First-Line Chemotherapy and TP53 Analysis

In the 62 patients of the control group, the results of the first-line chemotherapy showed a 51.6% overall response rate (28 partial responses and 4 complete responses). The median OS time was 9 months (95% Cls = 8–10.2 months). No significant association was detected between TP53 mutations and tumor response. Partial responses occurred in 5 patients (45%) with $TP53_{Inactive}$ missense mutations, in 13 patients (52%) with $TP53_{Active}$ missense mutations, in 2 patients (40%) with TP53 non-missense mutations, and in 8 patients (38%) without TP53 mutations. Complete responses were observed in one patient in each of the four groups. Median OS times were: 8 months (4.3–9.0 months 95% CI) in carriers of $TP53_{Inactive}$ missense mutations; 8 months (8.4–14.7 months 95% CI) in carriers of other TP53 mutations; 8.5 months (5.7–10 months 95% CI) in carriers of TP53Active missense mutations; 10.6 months (8.4–14.7 months 95% CI) in patients without TP53 mutations. A comparison of the survival curves using the log–rank test showed significant differences between the four groups (Figure 3). The analysis of hazard ratios with 95% CIs reveals a detrimental effect of the TP53Inactive missense mutations status in comparison to patients without TP53 mutations (Figure 3). The adverse effect of the $TP53_{Inactive}$ mutational status was retained in the multivariate model (Figure 2).

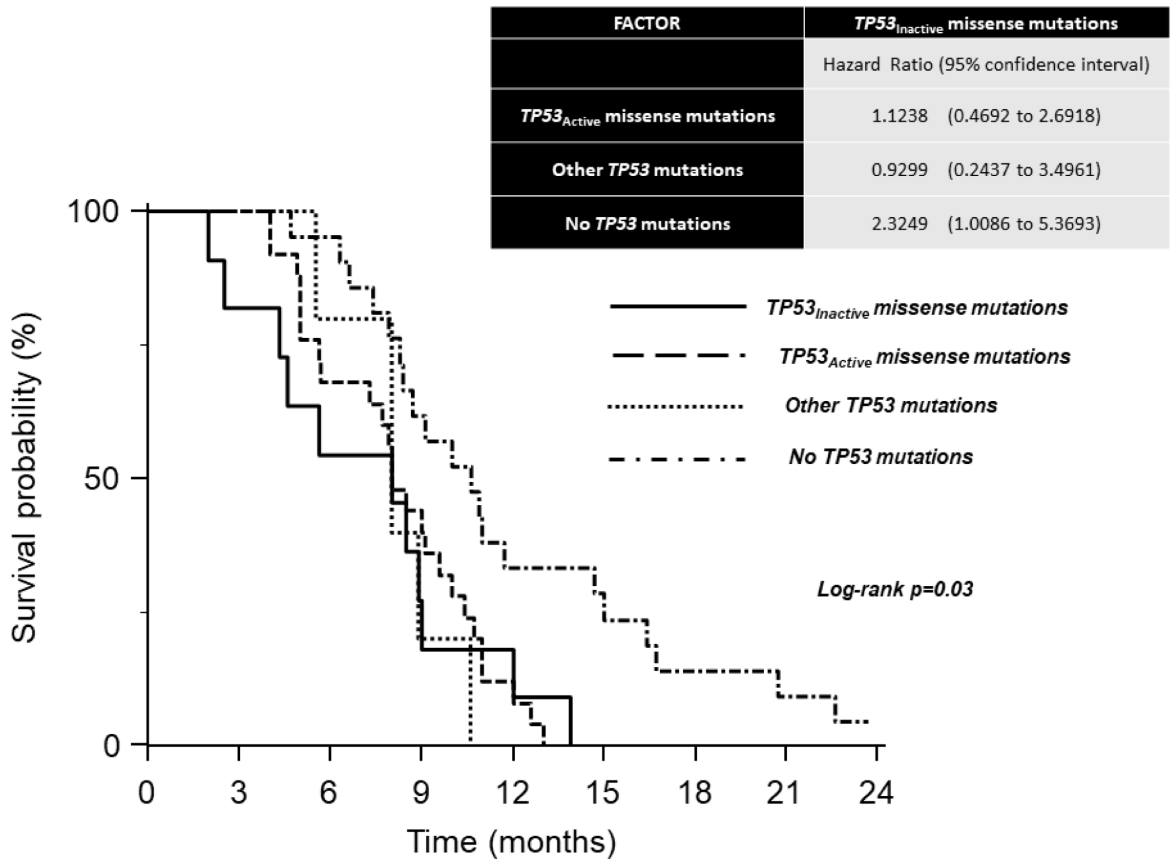

Fig.3 Kaplan-Meier survival curves of first-line combination chemotherapy in 62 patients with metastatic gastric cancer.

VEGF/VEGFR Analysis and TP53 Mutational Status in Gastric Cancer Tissues

Since Ramucirumab is a VEGFR2 antagonist that blocks the binding of VEGF-A, VEGF-C, and VEGF-D, we analyzed the mRNA expression and copy number alterations of these genes in gastric adenocarcinomas. VEGF-A gene gain was significantly more frequent in tumors with $TP5_{3Inactiv}$e mutations (58.1%) as compared to tumors with $TP53_{Active}$ mutations (35.7%) or wild-type p53 (13.4%) ($p = 0.019$ and $p < 0.0001$, respectively). Importantly, VEGF-A mRNA expression was correspondingly higher in the TP53Inactive group as compared to tumors with $TP53_{Active}$ or wild-type p53 ($p = 0.047$ and $p = 0.0039$, respectively). While no differences in the gene loss of VEGF-A were observed between these groups, the deletion of VEGF-C and VEGFR2 occurred less often in the wild-type p53 group as compared to the TP53 mutation subgroups ($p < 0.01$ and $p < 0.0001$, respectively), although this did not translate to significant differences in mRNA expression levels. Loss of VEGF-D occurred most frequently in the TP53Inactive group (32.6%), with significantly fewer deletion events in wild-type p53 tumors ($p < 0.0001$), however, there were no

differences in mRNA expression levels (Figure 4). Together, these findings support a mechanism exclusive to tumors with transcriptionally inactive p53 mutants, indicating a reliance on increased VEGF-A production to drive tumorigenesis.

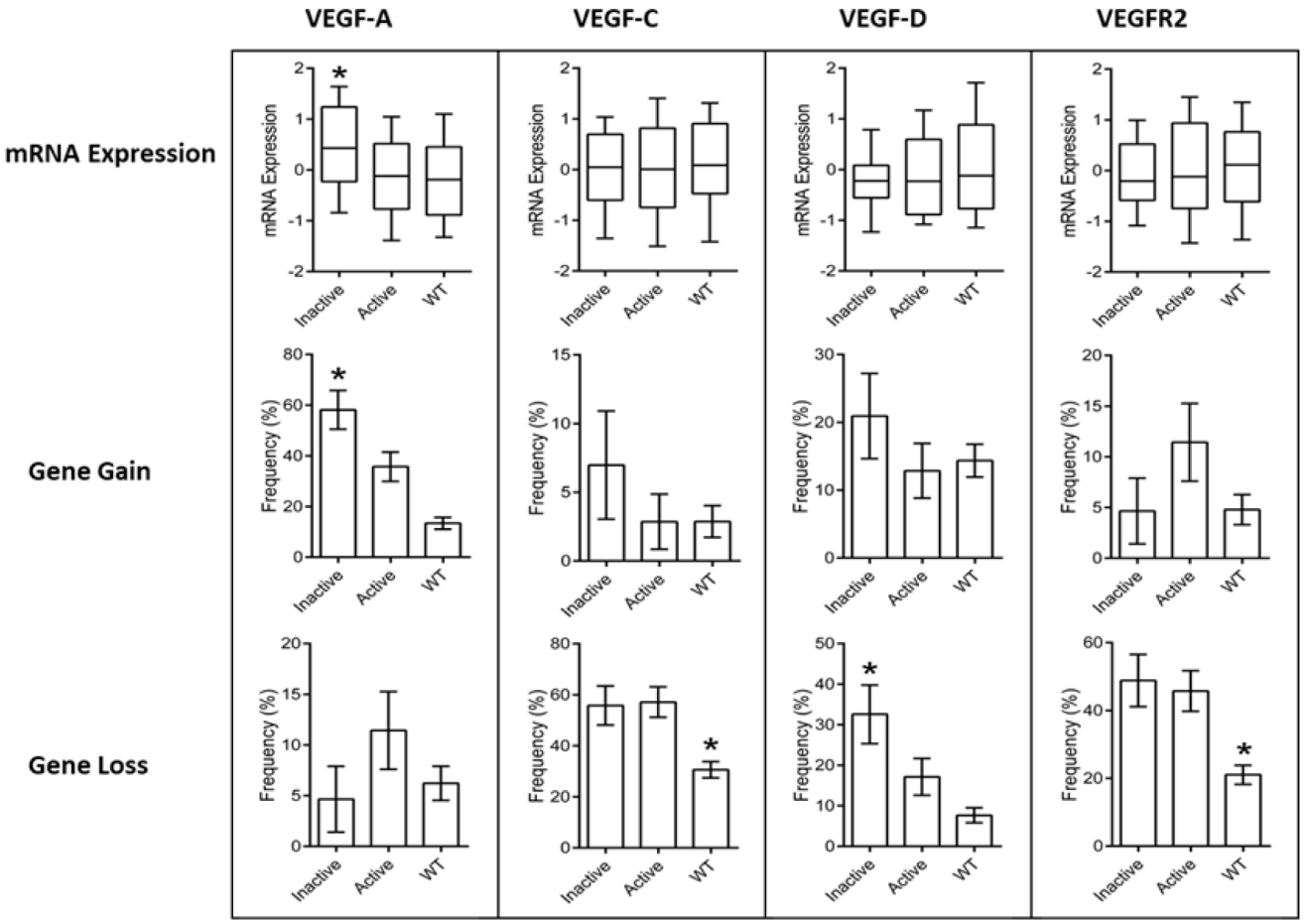

Fig.4 Plots of vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR2) analysis in gastric cancer tissues. data were collected from the TCGA PanCancer Atlas. * indicates statistically significant differences between groups as described in the text.

Discussion

The results of this study support the hypothesis that TP53 may be a valuable biomarker that can identify metastatic gastric cancer patients with the greatest benefit from an anti-angiogenic, anti-VEGFR2 systemic therapy. Importantly, the positive therapeutic effect, being associated with a specific group of transcriptionally inactive TP53 missense mutations ($TP53_{RTAS}$ < 1%) would simplify the development of a genetic test for further investigations, and hopefully, for routine clinical practice. This finding contributes to

a mounting body of evidence linking TP53 mutational status to anti-angiogenic treatment clinical outcomes in patients with advanced cancers [11–15]. So far, the loss of function of the TP53 tumor suppressor gene has been considered an unfavorable prognostic feature in patients with solid tumors [22]. Uncontrolled cell-cycle regulation, senescence, metabolism, and apoptosis in TP53 "null" neoplasms may explain this association [22]. However, the clinical impact of TP53 dysregulation may vary in patients undergoing anti-cancer systemic therapies, which could depend on differences in the mechanisms of action of anti-cancer agents [16]. Pre-clinical and translational studies have found links between TP53 loss of function and resistance to DNA damaging agents like platinum compounds and anthracyclines [16]. Conversely, tumors with loss of normal TP53 function may be even more sensitive to anti-cancer agents like Paclitaxel that stabilizes tubulin polymerization resulting in the arrest of mitosis and the induction of TP53-independent apoptosis [23,24]. It has been also demonstrated that Paclitaxel, especially in fractionated regimens, exploits anti-angiogenic mechanisms of action [25] Together, these chemotherapy-related aspects, in addition to pre-clinical and clinical studies linking TP53 mutations to the VEGF pathway [5–8] and anti-VEGF/VEGFR systemic therapies [9–15], contribute to explaining the favorable results of the Ramucirumab/Paclitaxel combination in metastatic gastric cancers harboring TP53 mutations. In the present study, we performed a combined analysis of $TP53_{RTAS}$ missense mutations and VEGF-A and VEGFR2 expression levels in gastric adenocarcinoma tumor tissue samples. The results indicate a significant VEGF-A up-regulation in tumor samples with $TP53_{Inactive}$ and unmodified VEGFR2 expression. These results parallel findings in previous analyses [3–7]. In a large pan-cancer cohort of 7525 samples, Li AM et al. [9] demonstrated up-regulated VEGF-A transcript levels in tumors with TP53 mutations, particularly in adenocarcinomas, regardless of their organ of origin, while VEGFR2 expression levels were not significantly modified by TP53 mutational status or reduced in squamous carcinomas. Since VEGF-A is considered the most potent angiogenic ligand and it exhibits the highest binding affinity for VEGFR2 [26], it is plausible that VEGF-A up-regulation is a major mechanism underlying the positive clinical impact of TP53 mutants on anti-VEGF/VEGFR2 therapies. Intriguingly, additional mechanisms may also explain the positive clinical interaction between chemotherapy, anti-angiogenics, and TP53 status.

In a translational analysis from a randomized trial in endometrial cancer, a remarkable survival benefit was found in the bevacizumab/chemotherapy arm in the presence of TP53 mutations causing loss of function or "null" phenotype [14]. Results from cell models suggested a mechanism of synthetic lethality derived from the effects of agents like bevacizumab to abrogate cell cycle checkpoints in the absence of p53 by blocking signaling downstream of tyrosine kinases [14]. This causes the premature entry of cancer cells into vulnerable phases of the cell cycle where chemotherapy agents are most effective. The majority of somatic TP53 mutations detected in human cancers are missense mutations [1,2]. These mutations, which arise from a point mutation in a single nucleotide, can result in amino acid changes that can lead to highly variable degrees of functional consequences. For example, an amino acid may be replaced by another amino acid with very similar chemical properties, resulting in a protein that still functions normally. In contrast, some amino acid changes may cause greater dysfunction or non-functional protein products. To overcome difficulties in the interpretation of TP53 mutational analysis, we adopted a functional classification of TP53 missense mutations based on a transcriptional activity score as the result of a site-directed mutagenesis technique and yeast-based functional assay [17,18]. Tumors harboring $TP53_{Inactive}$ missense mutations showed the longest survival time and the greatest benefit from the anti-VEGFR2 Ramucirumab/Paclitaxel systemic therapy. The analysis of survival curves suggests that tumors with TP53 non-missense mutations may also obtain some survival benefit from Ramucirumab/Paclitaxel (non-significant 52% risk reduction in the comparison with the wild-type group). Notably, TP53 non-missense mutants producing the loss of the protein product do not display specific properties of some missense mutations (i.e., hotspot mutants) with augmented oncogenic potential. This effect may be caused by their capacity to impair the wild-type allele (dominant-negative effects) and/or by specific gain-of-function effects [25,26]. Many missense TP53 mutants are expressed as stable proteins that exert dominant-negative effects by interfering with the remaining wild-type p53 protein copies through the formation of hetero-tetramers. A "prion-like" effect of some p53 mutants has also been shown to inactive wild-type p53 in vitro by forcing the wild-type protein to adopt a denatured, mutant-like conformation [2,27]. As a result of the gain-of-function effect, some TP53 missense mutants were found to promote

tumor angiogenic pathways, whereas TP53 deletion or truncating events did not [28,29]. Our analysis of TP53 mutations in relation to the clinical characteristics and pathological features of gastric adenocarcinomas in the present study population is supported by pivotal studies on the molecular classification of gastric cancer [30]. TP53 mutations characterize the most common of four molecular subtypes of gastric adenocarcinomas, defined by chromosomal instability. This genomically unstable subtype is associated with an intestinal histotype according to Lauren's classification, and a homogenous distribution along the different gastric sites.

Materials and Methods

The study group consisted of metastatic gastric cancer patients who received second-line systemic therapy with Ramucirumab 8 mg/kg (given on day 1 and 15) and Paclitaxel 80 mg/m2 (given on day 1, 8, and 15), both administered intravenously every 28 days. The cohort study was retrospective and performed among participating Institutions in the RAMoss analysis [31], which retrospectively evaluated the safety and efficacy of Ramucirumab among Italian patients failing first-line treatment for advanced gastric cancer. The control group comprised metastatic gastric cancer patients who underwent Cisplatin or Oxaliplatin plus 5-Fluorouracil systemic chemotherapy. This retrospective cohort was implemented from consecutive cases included in a large three-Institution database [32]. In both cohorts, the study inclusion required the availability of primary tumor tissue samples. The study was performed in accordance with the reporting recommendations for tumor marker prognostic studies (REMARK) guidelines [33]. All patient information and pathology materials were collected under a protocol approved by the Regional Ethical Committee (the protocol number is 2016-0374MN).

Samples and Nucleic Acids Extraction

A sample of 4–6 10-µm sections from formalin-fixed, paraffin-embedded specimens were obtained from patient tumors and matched normal tissues. Before cutting sections for total nucleic acid isolation, an additional slide was prepared for hematoxylin-eosin staining and the pathologists identified representative

areas with an almost complete representation of tumor infiltration. Tissues were micro-dissected and placed in a 1.5 mL reaction tube containing 1 mL xylene to remove paraffin. DNA was extracted using the RecoverAllTM Multi-Sample RNA/DNA Isolation Workflow (InvitrogenTM by Thermo Fisher, Foster City, CA, USA) according to the manufacturer's instructions. DNA concentration and purity were measured using the NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

Amplicons Library Preparation and Next-Generation Sequencing (NGS) for TP53 Analysis

A custom panel (IAD_119861) including the TP53 gene coding and UTR regions was designed using the Ion AmpliSeq™ Designer software (Thermo Fisher, Foster City, CA, USA). The panel was made up of 35 amplicons and ensured 82% of coverage for DNA from formalin-fixed paraffin-embedded (FFPE) tissues. Library preparation was performed using the Ion AmpliSeq Library Kit Plus according to the manufacturer's instructions. Libraries were generated using 40 ng of DNA from tumor FFPE sections and indexed using the Ion Xpress Barcode Adapter Kit. Library purification was carried out using the AMPureTM XP Reagent (Beckman Coulter, Brea, CA, USA) on the DynaMagTM-2 Magnet. QubitTM 4 Fluorometer (InvitrogenTM, by Thermo Fisher, Foster City, CA, USA) was used to quantify amplicons libraries. After dilution of all samples at 100 pM, libraries were pooled for emulsion PCR on the Ion OneTouch™ 2 instrument, using the Ion S5™ Template OT2 kit, according to the manufacturer's instructions. The Ion Sphere™ Particles were enriched using the Ion OneTouch™ Enrichment System and the template was sequenced on the Ion Torrent S5 platform using the Ion 540TM Chip (cat.no.A27766) following the manufacturer's instruction. All of these instruments and reagents were supplied by Thermo Fisher (Foster City, CA, USA). Read alignment was performed using hg19 (GRCh37) as the reference genome. Variant call files (VCF) were generated by the Variant Caller v.5 plugin preinstalled in the Torrent Suite and analyzed with the Ion Reporter™ software (Thermo Fisher, Foster City, CA, USA). BAM files were also manually checked on IGV (Integrative Genomics Viewer) [34].

Classification of TP53 Mutations

Each TP53 missense mutation was assigned a residual transcriptional activity score ($TP53_{RTAS}$) according to the results of a site-directed mutagenesis technique and yeast-based functional assay [17,18]. The $TP53_{RTAS}$ represents the median transcriptional activity value measured across eight different p53-responsive elements. Based on these functional scores, TP53 missense mutations were then divided into two distinct groups: TP53RTAS ≥ 1% and $TP53_{RTAS}$ < 1%. This categorization denotes a clear distinction between a transcriptionally inactive group ($TP53_{Inactive}$ =$TP53_{RTAS}$ < 1%) versus a transcriptionally active group ($TP53_{Active}$ =$TP53_{RTAS}$ ≥ 1%). Carriers of non-missense mutations including nonsense and frameshift mutations were merged into a third mutational group.

VEGF and VEGFR Analyses

A gastric adenocarcinoma dataset was collected from the TCGA Pan-Cancer Atlas (https://www.cancer.gov.tcga) for the analysis of mRNA expression, copy number alterations, and mutational data of genes of interest. Tumors with TP53 gene sequencing were selected and those with more than one TP53 alteration were excluded. Individual tumors were then assigned a TP53 mutation-specific RTAS, sub-grouped based on the RTAS, and analyzed for the gene expression and copy gain or loss of VEGF-A, VEGF-C, VEGF-D, and VEGFR2.

Statistical Analysis

The primary endpoint of the study was the overall survival (OS) analysis in carriers of $TP53_{Inactive}$ mutations in the Ramucirumab/Paclitaxel study group. With 40 events and a 20% prevalence of the $TP53_{Inactive}$ mutational status, the scenario for sample size estimation would allow detection of a 66% reduced risk of death with a power of 80% and a two-sided type I error of 5%. In the Ramucirumab/Paclitaxel group, OS was calculated from the date of the first cycle of the second-line therapy to the date of death or last follow-up. In the chemotherapy control group, OS was calculated from the date of the first cycle of the first-line therapy to the date of death or last follow-up. The Kaplan–Meier method was used to estimate

survival curves and the log–rank test was used to compare survival times between groups. A multivariate Cox proportional hazards model was adopted for adjusting according to clinical and pathological features. Patients achieving complete response or partial response and patients with stable disease or disease progression were evaluated according to the RECIST criteria and the overall response rate included patients with a complete response and partial response. Contingency tables were analyzed by the Chi-square test. All reported p-values were two-sided, and confidence intervals (CIs) were at the 95% level. A p-value <0.05 was considered statistically significant. Survival analyses were performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium). Data processing for the VEGF/VEGFR analyses in gastric cancer tissue was completed using R statistical environment version 3.6.2 and figures were generated using GraphPad Prism version 6.07.

Conclusions

The limitation of this study is the relative sample size, so our findings warrant further investigations to confirm the association between transcriptionally inactive TP53 missense mutations and improved clinical outcomes of patients with metastatic gastric adenocarcinoma who received anti-VEGFR2 plus Paclitaxel systemic therapy. From a clinical perspective, the $TP53_{RTAS}$ mutational analysis might improve the identification of patients who are likely to have the greatest benefit from Ramucirumab therapy. Ramucirumab and chemotherapy failed to achieve significant survival advantages in a randomized phase III study when adopted as a first-line therapy for metastatic gastric cancer [35]. In the overall treatment strategy for the metastatic disease, the selection of patients according to $TP53_{RTAS}$ mutational status represents a promising model to tailor treatment choices and improve clinical outcomes. In addition, $TP53_{RTAS}$ analysis could be evaluated in patients with metastatic adenocarcinomas in other solid tumors with frequent TP53 mutations and where anti-VEGF therapy is commonly employed.

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CONCLUSIONS

Gastric cancer (GC) is one of the most common cancers worldwide and ranks second in cancer-related deaths. Although there are many therapeutic approaches available for GC most patients die shortly after diagnosis because of the high inter- and intra-tumor heterogeneity, and because the majority of patients are diagnosed with the metastatic disease. Resistance, both intrinsic and acquired, to the novel anti-angiogenic-based therapies is an additional concern in the GC management. Several studies showed that metabolic reprogramming with adaptive escape in response to a hypoxic tumor microenvironment may explain, at least in part, the failure of anti-angiogenic treatments in solid tumors.

To gain survival advantage, tumor cells are able to switch metabolism from oxidative phosphorylation to aerobic glycolysis (Warburg effect). Recent translational clinical studies in cancer patients sustain the putative clinical impact of Warburg effect-related proteins on prognosis, and there is mounting evidence that these features negatively affect survival outcomes. [27]

Little is known about this phenomenon in GC patients treated with anti-angiogenic regimens, so we planned a study to evaluate the possible predictive impact of glucose metabolism deregulation on clinical outcomes. Due to the role of *TP53* in the control of glycolysis and the incidence of *TP53* mutations in GC, we also analysed the *TP53* mutational status to assess if it may influence the metabolic reprogramming. The gene expression analyses of five glycolysis-related genes (*GLUT1*, *PKM2*, *LDHA*, *HK1* and *HK2*) were performed by RT-qPCR comparing the expression level profiles in the tumor mucosa with the normal mucosa in 40 metastatic GC patients treated with Paclitaxel-Ramucirumab (PR) systemic therapy. We found that all targets tested, except for *HK1*, were up-regulated in tumor mucosa towards normal mucosa. Then patients displaying the contemporary up-regulation of three key glycolytic enzymes were categorized as Positive Glycolic Profile (PGP), where the remaining patients were categorized as Negative Glycolytic Profile (NGP). According to PGP and NGP profiles, we found 18 (45%) PGP cases, displaying a survival advantage under anaerobic conditions and associated with significantly worse progression-free and overall survival compared to NGP cases. In this setting of patients we performed an additional analysis where we evaluated the *TP53* mutational status by Next-Generation Sequence (NGS). Surprisingly, *TP53* mutations were almost equally distributed between PGP and NGP groups, without any significant association with

clinical outcomes. Even in a small sample size, these findings point out that glycolytic competence may negatively affect survival outcomes of metastatic GC patients treated with PR systemic therapy. Further investigation is needed to confirm our findings that would promote the development of novel therapeutic strategies against cancer metabolism useful to improve the efficacy of PR therapy.

In the second study we characterize the possible predictive impact of *TP53* mutations on PR therapy compared to standard chemotherapy in 110 metastatic GC patients. Classifying mutations on the basis of the residual transcriptional activity score (RTAS), we observed that $TP53_{\text{inactive}}$ mutations differentially affect survival outcomes depending on the anti-cancer regimen. More in detail, PR-treated patients displaying $TP53_{\text{inactive}}$ mutations showed a better overall survival respect to patients carrying $TP53_{\text{active}}$ mutations. Conversely, in the standard chemotherapy group, patients with $TP53_{\text{inactive}}$ mutations showed the worst overall survival. Moreover, *VEGF* expression levels were significantly increased in $TP53_{\text{inactive}}$ cases. This phenomenon may be explained, at least in part, by the influence of p53 on *VEGF* expression. In fact, p53 abrogation increases *VEGF* expression levels and thus improves efficacy of anti-VEGF/VEGFR therapies, such as PR.

Our results suggest that $TP53_{RTAS}$ mutational analysis may be helpful in the identification of patients that probably will benefit most from Ramucirumab therapy.

Due to the limitations of the study, such as its retrospective nature and the relative small sample size, further investigation is necessary to confirm the hypothesis that $TP53_{RTAS}$ mutational status may represent a promising model to tailor treatment choices and improve clinical outcomes in metastatic GC patients.

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Chapter 1

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Chapter 2

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