UNIVERSITÀ DEGLI STUDI DI PALERMO
Dottorato di ricerca in Oncologia e Chirurgia Sperimentali
Dipartimento di Discipline Chirurgiche Oncologiche e Stomatologiche (Di.Chir.On.S.)

LncRNA H19, HOTAIR AND MALAT1 AS MOLECULAR BIOMARKERS IN GIST

Doctoral Dissertation of:
Nadia Barraco

Supervisor:
Prof. Paolo Vigneri
Prof. Juan Iovanna

Tutor:
Prof.ssa V. Bazan

The Chair of the Doctoral Program:
Prof.ssa Giuseppina Campisi

Years 2014/2016 – Cycle XXIX

INDEX

Abstract

Long non-coding RNAs (lncRNAs) are emerging as essential regulators of genetic and epigenetic networks, and their deregulation may underlie complex diseases, such as carcinogenesis. Several studies have described lncRNAs alterations in patients with solid tumors. In particular, in Gastrointestinal Stromal Tumors (GIST), upregulation of HOTAIR has been associated with tumor aggressiveness and metastasis, and poor patients' survival. In order to gain more detailed insight on the molecular role of lncRNAs in GIST, we analyzed *in vivo* the expression levels of lncRNAs H19, HOTAIR and MALAT1 in tissue specimens of both surgically resected and metastatic GIST patient. The expression of the lncRNAs H19, HOTAIR and MALAT1 was evaluated in a total of 40 patients with GIST using quantitative real-time reverse transcriptase (qRT-PCR). H19 was overexpressed in 50% (20/34) GIST patients (p-value: 0.0496). MALAT1 was overexpressed in 45,15% (14/31) GIST patients (p-value: 0.032). Furthermore, the up-regulation of H19 has been found in 74% (17/23) patients harboring cKIT mutations compared to 4/7 (57%) wild type patients (p-value: 0.042). Conversely the up-regulation of MALAT1 has been found in 76% (13/17) patients harboring cKIT mutations compared to 4/4 (100%) wild type patients (p-value: 0.027). Finally, the up-regulation of H19 has been found in 100% (5/5) patients with TTP < 3 months compared to 25% (1/4) patients with TTP >3 months, while the up-regulation of MALAT1 has been found in 25% (1/4) patients with TTP < 3 months compared to 75% (4/5) patients with TTP >3 months.

H19 and MALAT1 appear upregulated in GIST patients according to the KIT-mutation status. These data would suggest a potential, opposite prognostic value of both H19 and MALT1 lncRNAs in these patients. Further analyses are needed to confirm these data, and evaluate the potential role of such lncRNAs, as prognostic/predictive biomarkers.

CHAPTER 1

Background Rationale and Objectives

1.1 Introduction

"We are more than the simply sum of our genes". Over the last decade, this is the idea that characterized the post-genomic era and it will likely allow us to understand the molecular cellular identity. Indeed every type of cell shows the same single genome but different epi-genomes able to determine the final cellular identity[4]. Therefore the "personality of cells" becomes definite by its epigenome that is the result of both the classic Mendel's laws of genetic inheritance and epigenetic, whose basic unit is the nucleosome[5,6]. The nucleosome structure together with non-histone proteins and RNAs constitute the plasticity of chromatin architecture (Figure 1). Indeed, the set of ATP-dependent chromatin remodeling complexes covalent modifiers, and non-coding RNAs, seem to play a key role in the network of epigenetic regulation being able to make modification beyond the DNA sequences[7]. Although these events take place physiologically, it was also observed that aberrations arising at RNA or protein levels, may influence the tumor phenotype without generating any changes in genes coding[8,9] (Figure 2). Recent data have shown that several emerging classes of non-coding RNA, such as HOTAIR, Xist ed AIR long non coding RNA (lncRNA) interact with chromatin remodelling complexes in order to direct themselves toward gene targets[10-13]. LncRNAs are emerging as essential components of gene regulatory networks, including cancer[14]. In the current scenario, characterized by a spasmodic research of new biomarkers and the advent of advanced technologies, the lncRNAs represent a new, valid and largely unexplored field of investigation, that I like wildly think as "super-natural or epi-DNA".

1.2 The little world of long non-coding RNA (LncRNA)

It is the versatile molecule of RNA that, always considered as a simple mediator between DNA and proteins, in the last decades has received favorable consensus, being recognized as a centerpiece of the complex world that goes around the life of the cell[15,16]. Indeed, recently the scientific community has re-considered all the potential scenarios in which the lncRNAs and their related mechanisms of action could shed light on organismal complexity[17,18]. LncRNAs belong to a class of regulatory RNA noncoding for proteins that, as it has been estimated, represent approximately 1.5% of the eukaryotic genome, almost entirely transcribed[19-21]**.** The NONCODE human lncRNA database annotated 527,336 transcripts that are antisense, intergenic, sense intronic, and processed transcript[22]. Differently from toother transcriptome and epigenome data sets the lncRNAs are generated through a molecular pathway similar to that used for protein-coding genes [23]. LncRNAs have been arbitrary defined according to their size, as transcribed RNA molecules greater than 200 nt in length in their mature form. In contrast to the small ncRNAs (siRNAs, miRNAs, and piRNAs), which are highly conserved in commonly studied species, and act as negative regulator of gene expression, lncRNAs are modestly conserved, and regulate gene expression through mechanisms that are mostly poorly understood[24-28].

1.3 LncRNAs as subject: features and functions

As mRNA, lncRNAs are often multi-exonic[21] and lack an open reading frame of significant length (less than 100 amino acids)[29-31]. LncRNAs are preferentially localized in the chromatin and nuclear RNA fractions, but also in cytoplasm fractions. They may have or not 3'poly(A) motif and 5' capped, showing canonical splices sites. LncRNAs are subjected to transcriptional and post-transcriptional regulation, thus lacking any protein-coding potential[23,32]. Despite the majority of lncRNAs' functions remain unknown, it seems that their effects are likely due to a synergistic interaction with other components[33-35]. Indeed their peculiar structure allows them to easily interact with DNA, RNA nucleic acids and proteins[36,37]. Thus far, it has not beenclarified yet the specific molecular mechanisms used by lncRNAs to regulate such processes and if they play an active or a passive role in this context[21]. Thanks to their structure, they play a

critical role in a plethora of biological functions at transcriptional, post-transcriptional and translation levels, including also epigenetic processes [27,28,35,38].

This wide range of cellular functions are not mutually exclusive[21] (Figure 3).

- ✓ As signal, the expression of lncRNAs responds to intra- and extracellular stimuli in a specific space-time (Figure 3 I).

In mammals, lncRNAs can act as signals for the regulation of gene expression during the development process, or in stress conditions, or during the splicing of pre-mRNA. They can recruit chromatin-modifying factors on their target gene promoters inducing transcriptional active euchromatin or silent heterochromatin status. They can also contribute merely in a signaling pathway[21,39].For example, the overexpression or down-regulation of lincRNAs induces high or low levels of induced pluripotent stem cells (iPSCs), respectively, and their absence is associated with dysregulation of p53, in response to a particular stress as DNA damage (confirming the key role of lincRNAs in the induction of pluripotency)[40].

- ✓ As Decoy, lncRNAs tighten the interaction with ribonucleoproteins structure (RBPs) on chromatin, into nuclear subdomains or in the cytoplasm[21,41] (Figure 3 II).

LncRNAs can recruit transcriptional factors, chromatin modifiers or other regulatory factors[21]. Also, they can interact with DNA to cause a triple helix structure that block the maturation of pre-initiation mRNA complex[42]. Instead, at post-transcriptional level, lncRNA-mRNA double helix complex can grow the stability of mRNA and it may prevent the degradation of mRNA [43]. Furthermore, lncRNAs can bind regulatory microRNAs[44]. The interaction microRNA-lncRNA can cause the degradation of lncRNAs or can promote the creation of small microRNA. Thanks to their “miRNA-sponge” action lncRNAs can block the microRNA function or may act as endogenous competitor for the binding with mRNA targets[45,46] (not shown on figure).

- ✓ As Guide, lncRNAs form RBP complexes in *cis* or in *trans* position of their production site (Figure 3 III).

lncRNAs can bind the proteins involved in the transport activity likely to facilitate the localization in the specific chromosome sites or to recruit chromatin-modifying enzymes

- ✓ As Scaffold, in the same space and time (Figure IV),

lncRNAs can bring together several different proteins, to make cellular substructures or protein complexes that has an effect on chromatin inside or outside the cell [18].

So, they take part to both local and global changes in chromatin packaging.

Since lncRNAs regulate several biological processes, their overexpression may be essential in the switch towards pathological conditions[43,47]. They may be involved in the development of different human diseases[48,49]such as cancer[28,49,50]. They may act as pro-oncogenic and/or tumor suppressor factors, modulating both tumor initiation, progression and metastatic pathways[10]. The majority of validated hypothesis suggested that the aberrant expression of lncRNAs related to the activation of oncogenes such as Myc and p53[47].

Experimental evidences suggested that dysregulation of lncRNAs promotes tumorigenesis and metastasis of several human cancers (Figure 4). Among the different lncRNAs identified in solid tumors, H19, HOTAIR and MALAT1 represent those better studied and characterized as described below [12,27,51].

1.4 LncRNA H19

LncRNA H19 has been one of the first to be identified. It is mostly expressed in the embryonic and fetal tissues, but its expression is reduced in adult tissues[52]. The H19 locus encodes a 2.3 kb noncoding and it maps on 11p15.5 chromosome in humans. This gene belongs to a subgroup of imprinted genes. The loss of *H19* maternally expressed gene and its consecutive expression alteration has been observed in different solid tumors[53]. qRT-PCR analyses on gastric cancer cells and tissues, obtained during surgical resection, has shown a higher expression of lncRNA H19 than that observed in adjacent normal gastric cancer (GC)[54], non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), ovarian cancer (OC), gallbladder cancer (GBC), laryngeal squamous cell cancer (LSCC), colorectal cancer (CRC), esophageal cancer (EC) and hepatocellular carcinoma (HCC) tissues. Its

dysregulation has been correlated with worse survival, poor DFS, histological grade, positive lymph node metastasis and advanced TNM stage[55]. Also, in plasma of pre-operative patients with GC, the H19 plasma levels were high far more than healthy control patients although in no way there were conformity with clinico-pathological characteristics. It's different the situation/analyses using paraffin-embedded tissues. There was no different H19 expression levels between cancerous tissues and paired non-cancerous tissues[56]. Despite the molecular mechanisms remain unclear, in GC tissue and cell lines, H19 acts rarely as tumor suppressor[57,58], more as an oncogene through its mature product mir-675[59,60]. They directly or indirectly act on different target genes modulating different molecular pathways. Probably, the activation of Akt/mTOR pathway for the progression of GC seems to be mediated by inactivation of tumor suppressor RUNX1 (runt domain transcription factor 1), which is a target of mir-675[61,62]. Even the inactivation of p53 seems to grow the H19 expression level in hypoxic condition, likely through HIF1a, supporting the proliferation of gastric cancer cells[54]. The consequent epithelial–mesenchymal transition (EMT) phenomenon may be responsible of metastatic phenotype and/or pharmacoresistance events[63]. From a meta-analysis results, high H19 level expression was inversely correlated with OS and prognosis in many types of cancer, that could make it a negative prognostic biomarkers[55].

1.5 LncRNA HOX antisense intergenic RNA (HOTAIR)

HOX antisense intergenic RNA (HOTAIR) encodes a large intergenic lncRNA. It is located within the HOXC locus in antisense orientation relative to the HOXC genes. Thanks to its peculiar archytecture it plays a key role as scaffold for the formation of different complexes. HOTAIR consists of two modules and a linker sequence. Both this modules bind a chromatin remodelling factor at target locus: the Polycomb Repressive Complex 2 (PRC2) and the lysine-specific demethylase 1 (LSD1), promoting the gain of trimenthylate lysine 27 of histone H3 (H3K27me3), which is a well-known repressive mark, and the loss of H3K4me2methylation, considered asan active trascription mark. Thereby, it is able to establish the silent eterochromatin state (essential to silence the locus HOXD located in trans on a different chromosome[64] to alter other genes' expression patterns[65]. HOTAIR is considered a prognostic unfavorable marker in various kinds of cancer despite there has been not any significant association between

its abnormal expression and the clinical-pathological features of analyzed patients. Higher HOTAIR expression has been observed in breast, gastroenteric, liver, lung, prostate, pancreatic, sarcoma cancer tissues compared to the adjacent normal tissues[12,66]. In some tumor types higher levels of HOTAIR have been detected in plasma of cancer patients[66] compared to healthy controls, but in GC patients the plasma levels of such lncRNA) were similar to the normal population[56].

1.6 LncRNA MALAT1

MALAT1 is the most expressed within the lnc transcripts. Its transcript is long 6.7 kb. It localizes into nuclear speckles[67-69] reaching the citoplasmatic compartment as RNA of few nt[70]. Nuclear-localized lncRNA MALAT1 shuttles to transcription start sites where it functions as scaffold to guide transcription and alternative pre-mRNA splicing in a target gene. It acts as oncogenic factor. In stress condition it has been found upregulated in common site of metastasis from different solid tumors first of all lung cancer[71]. In this type of tumor it plays a role as negative prognostic marker [72,73]. As shown by a recent meta-analysis, also in breast, ovarian, colon, pancreatic and digestive cancer, the high expression of MALAT1 positively correlated with worse patients' prognosis while in B cell lymphoma the condition was the opposite. Indeed, high level of MALAT1 1 expression was associated to a good patients' prognosis[74]. Similarly to HOTAIR, MALAT1 plasma levels in GC patients were no different compared to the healthy control [56].

1.7 LncRNAs and GIST

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor belonging to the class of sarcoma. We usually find them in gastrointestinal tract at a percentage of 60-70% in the stomach or even of 30% in small bowel, with lower frequency up to esophagus. GISTs can develop sporadic mutations in the c-KIT receptor and platelet-derived growth factor receptor alpha (PDGFRα) proto-oncogenes, which encode both KIT and PDGFRα proteins, respectively. Mutations of c-KIT exons 9/11 are reported in about 80 % of GISTs, making them sensitive to the targeted therapy with the multi-target tyrosine kinase inhibitor (TKI) imatinib mesylate, whose advent has revolutioned the natural history of such disease. Conversely PDGFRα mutations are described in about 5-8% of GISTs and are associated with the

occurrence of primary resistance to the TKI imatinib. Finally about 15% of all GISTs don't report any mutations in both c-KIT and PDGFRα genes and are defined as "wild-type". For GIST patients with localized disease who underwent the surgical treatment, the evaluation of cKIT/ PDGFRα molecular alterations together with other clinical-pathological factors, including tumor size/site, mitotic rate, and proliferation index, is crucial to predict the potential risk of recurrence and ultimately decide if patients are candidate to receive adjuvant therapy with imatinib. As regards patients with advanced disease harboring c-KIT activating mutations imatinib mesylate represents the standard first-line treatment, while sunitnib and regorafenib are two multi-target TKIs usually administered after imatinib failure. Currently both c-KIT and PDGFRα mutations represent the only approved molecular biomarkers taken into account by clinicians to decide the medical therapy. In this scenario the identification of lncRNA as new potential diagnostic, prognostic, and predictive molecular biomarkers represent a new challenge for current translational research, especially in rare tumors such as GISTs[75]. Few working groups have studied, *in vivo* and *in vitro*, the expression of lncRNAs in GISTs. Niinuma et al. in 2012 described the deregulation of the HOTAIR expression in GIST patients. In particular, HOTAIR was up-regulated in high-risk malignancy samples from frozen GIST tissues. The lnc-RNA tissue overexpression was associated with metastasis and poor overall survival, even it was not statistically significant. This analysis was not confirmed in FFPE specimens, likely due to the low quality of RNA. *In vitro*, knockdown of HOTAIR in GIST-T1 cells influences the expression of target genes and the invasive ability of cells though not the cell viability. The dysregulation of both HOTAIR and other downstream genes expression in cancer cells, need to be ascribed to an epigenetic mechanism. Looking to the chromatin status of GIST-T1 cells, the histone codes show an enrichment of a marker of active gene transcription, H4K4me3, in the transcription start sites of genomic regions of interest[76]. Alternatively, the overexpression of HOTAIR can mediate the trimethylation of the histone H3 at lysine 27 and the subsequent silencing of the target gene. An additional study by Lee et al. has recently confirmed such evidences, showing that if the target gene subjected to silencing is a tumor suppressor as PCDH10, the final result will be the failure of the mechanisms which control both tumor invasion and progression[77]. Even if very interesting, these are the only published data currently available regarding lncRNAs expression in GISTs.

1.8 Rationale and Objectives

An increasing number of studies described lncRNAs expression alteration in different solid tumors, suggesting their potential involvement in tumorigenesis and metastatic processes, as well as their role as prognostic/predictive biomarkers for clinical use. However very few data are currently available on lncRNAs dysregulation in rare tumors such as GISTs. Our work represents one of the few studies evaluating lncRNAs expression in GISTs. Indeed, in order to gain more detailed insight on the molecular role of lncRNAs in GIST, we analyzed *in vivo* the expression levels of lncRNAs H19, HOTAIR and MALAT1 in tissue specimens of both surgically resected and metastatic GIST patients. Identifying reliable biomarkers of both cancer risk development/recurrence and prognosis, and treatment sensitivity/resistance predictors, represents an area of intense investigation in the cancer research. Therefore we subsequently tried to assess the potential prognostic role of these lncRNAs in relation to other clinical-pathological parameters in GIST patients as well as the possible association between their upregulation and the targeted therapy efficacy.

CHAPTER 2

Materials and Methods

2.1 Study population

From January 2009 and November 2016 a total of 40 patients were enrolled within a single-institutional translational research study at the Oncology Department of Palermo University Hospital, including 35 patients with localized disease and 15 patients with advanced disease. Written informed consent was obtained from all patients before inclusion in such study and specimens collection. All patients with diagnosis of metastatic disease harboring cKIT activating mutations received oral imatinib mesylate at 400/800 mg daily until progression (PD) or unacceptable toxicity. Among the patients with localized disease subjected to surgical treatment, only those defined at high risk of recurrence according to the risk definition system proposed by Miettinen's et al.[78] received oral imatinib mesylate at 400/800 mg daily for 3 years. All the patients underwent a CT-scan every 3 months and responses were classified according to RECIST criteria. Clinical and pathological characteristics of all GIST patients included in our study were retrieved from the clinical records available and were assessed retrospectively.

2.2 Tumor samples

A total of 40 pairs of disease formalin-fixed paraffin-embedded (FFPE) tissue and adjacent normal tissue from 40 patients with GIST were collected from Pathology Department of Palermo University Hospital between 2009 and 2016. Resected GISTs specimens were fixed in buffered formalin and embedded in paraffin for pathological examination. The same standard methods were used for pathological assessment of

the tissue biopsies from patients with metastatic onset. After that all sample sections (10 μm thick) were received at the laboratory of Genetic and Clinical Molecular Oncology, where they were subjected to the molecular analysis of KIT exon 9, 11, 13, 17 mutations and PDGRFa exon 12, 14 and 18 mutations. Furthermore, we have obtained other sections of the tumor and simultaneously of the countparters healthy, which were used for gene expression analysis.

2.2 DNA preparation and mutation screening

Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections using a QIAamp DNA FFPE Tissue Kit (Qiagen). To detect hotspot mutations, we amplified exons 9, 11, 13, 17, and 18 of the *KIT* gene by PCR in a preparation of genomic DNA. The primer sequences are listed in Table 2. We purified PCR products with PureLink® PCR Purification Kit (Thermo Fisher SCIENTIFIC), and directly sequenced them using BigDye XTerminator® Purification Kit (Thermo Fisher SCIENTIFIC) on an ABI 3130 XL Genetic Analyzer automated sequencer (Applied Biosystems). Sequence data were analyzed using Sequencing Analysis software 5.2 (Applied Biosystems).

2.3 lncRNA isolation

Formalin-fixed, paraffin-embedded tissues samples were deparaffinized and underwent total RNA and lncRNA extraction using miRNeasy FFPE Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. LncRNA yield was determined through a Qubit™ 3.0 Fluorometer (Thermo Fisher SCIENTIFIC), and the quality assessed by agarose gel electrophoresis. The lncRNA concentration and quality were assessed with the Bioanalyzer 2100 (Agilent Technologies, CA) using the Agilent Small RNA Analysis kit (Agilent, CA).

2.4 Reverse Transcription (RT) and quantitative Real time Polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR was used to measure lncRNA expression levels in 40 disease/normal-paired GISTs samples. 500 nanograms of total RNA were reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher SCIENTIFIC) according to manufacturer's instructions. RT reactions contained RNA sample, 1 × 1 mL of 10X RT Buffer, 1 × 1 mL of 10X RT Random Primers, 1 × 0.2 mL of

25X dNTP Mix (100 mM), 2 × 0.1 mL of MultiScribe® Reverse Transcriptase (50 U/μL) (all from ThermoFisher SCIENTIFIC) and nuclease-free water. The 50-μl reactions were incubated in a Thermocycler (Eppendorf, North Ryde, New South Wales, Australia) for 10 min at 25°C, 120 min at 37°C, 5 min at 85°C and then held at 4°C.

The following Applied Biosystems assays were used for TaqMan analysis of *H19* (Assay ID Hs00399294_g1); *HOTAIR* (Assay ID Hs03296631_m1); *MALAT1* (Assay ID Hs00273907_s1) as potential prognostic biomarkers. The reactions were incubated in a 96-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantitative PCR was performed on an Applied Biosystems 7900HT fast RT-PCR system, and data were collected and analyzed using ABI SDS version 2.3. Triplicate reactions were performed on all samples. To normalize qRT-PCR reactions, parallel reactions were run on each sample for *GAPDH* (Assay ID Hs03929097_g1). Changes in lncRNA expression levels were determined using a comparative CT method.

2.5 Statistical Analysis

Statistical analysis was performed using Microsoft Excel and Prism GraphPad software (GraphPad software, CA).

A Fold Change > 1 (logarithmic scale) cut-point was used to define lncRNA up-regulation in analyzed tumor tissues.

Time to progression (TTP) was calculated from the date of admission to the date of first radiologic progression. Survival analysis was performed using Kaplan–Meier method, providing median and 95% confidence interval (CI)

To compare two independent samples, X^2 test was used for intergroup comparison of categorical variables, while the Mann Whitney test was used for statistical analysis of continuous variables. A p-value < 0.05 was used as a threshold for statistical significance

CHAPTER 3

Results

3.1 Clinical characteristics of the patients

Forty patients with histopathological diagnosis of GIST were included into the study. Median age of the patients was 59 years. Majority of the patients were male (n.26, 65%). More than half (n.25, 64%) of the cases had localized disease at diagnosis, so 36% represented with stage IV disease. C-KIT mutations were detected in 27/40 (67%) of patients, 17/25 patients with localized disease and 10/15 patients with metastatic disease at baseline. Consequently 8/25 (32%) patients with localized disease harboring c-KIT mutations received adjuvant treatment with imatinib for 3 years because of high risk of relapse. Conversely 10/15 (66%) patients with metastatic disease received first-line therapy with imatinib mesylate until PD or unacceptable toxicity. Clinical and pathological characteristics of patients included in the study are summarized in Table 1.

3.2 Expression of lncRNAs H19, HOTAIR, MALAT1 in GISTs

Among the 40 patients included 34 were evaluable for lncRNA H19 expression analysis in tumor tissue. As shown in figure 5 the up-regulation of H19 has been found in 20/34 (50%) patients with GIST with median $\log_2$ fold-change of 5 (Table 3). Furthermore the up-regulation of H19 was significantly higher in tumor tissue of GIST patients than in normal tissue of the same individuals with p-value: 0.0496.

Among the 40 patients included 31 were evaluable for lncRNA MALAT1 expression analysis in tumor tissue. As shown in figure 6 the up-regulation of MALAT1 has been

found in 14/31 (45,15%) patients with GIST with median fold-change of 4 (Table 3). Furthermore the up-regulation of MALAT1 was significantly higher in tumor tissue of GIST patients than in normal tissue of the same individuals with p-value: 0.032.

Among the 40 patients included none of them was evaluable for lncRNA HOTAIR expression analysis in tumor tissue (Table 3).

No statistically significant difference has been detected for the up-regulation of both lncRNAs H19 and MALAT1 in relation to the different clinical-pathological characteristics analyzed, including site/size of tumor, mitotic index, stage at diagnosis, and risk stage according to Miettinen's criteria (Table 4, 5).

A statistically significant difference has been detected for the up-regulation of both lncRNAs H19 and MALAT1 between the KIT-mutated and wild type tumors. Indeed the up-regulation of H19 has been found in 17/23 (74%) patients harboring cKIT mutations compared to 4/7 (57%) wild type patients (p-value: 0.042) (Table 4). Conversely the up-regulation of MALAT1 has been found in 13/17 (76%) patients harboring cKIT mutations compared to 4/4 (100%) wild type patients (p-value: 0.027) (Table 5).

3.3 Relation of lncRNA upregulation and treatment efficacy

To investigate the predictive value of both lncRNAs H19 and MALAT1 in GIST patients we evaluated the up-regulation of such lncRNAs in tumor tissues of 10/40 patients with advanced disease who received first-line therapy with imatinib.

A statistically significant difference has been detected for the up-regulation of both lncRNAs H19 and MALAT1 between patents with early PD compared to those with late PD. Indeed the up-regulation of H19 has been found in 5/5 (100%) patients with TTP < 3 months compared to 1/4 (25%) patients with TTP >3 months (Table 4). Conversely the up-regulation of MALAT1 has been found in 1/4 (25%) patients with TTP < 3 months compared to 4/5 (75%) patients with TTP >3 months (Table 5).

Furthermore a statistically significant difference has been detected for the median TTP between patients with lncRNA H19 up-regulation vs those with lncRNA H19 down-regulation (7,5 weeks vs 22,5 weeks; p-value: 0.025) (Table 4). Similarly a statistically significant difference has been detected for the median TTP between patients with

lncRNA MALAT1 up-regulation vs those with lncRNA MALAT1 down-regulation (15 weeks vs 3 weeks; p-value: 0.036) (Table 5).

CHAPTER 4

Discussion

Several evidences identified lncRNAs dysregulation in tumor pre-clinical models, suggesting their potential involvement in cancer development[79,80]. We have evaluated the expression of H19, HOTAIR, MALAT1 because theywere better studied among the different lncRNAs identified in solid tumors as an hallmark of poor prognosis. They contribute to oncogenesis in cancer as oncogenic and/or tumor suppressor factors[81]. They play essential biological functions including chromatin modification, transcriptional and post-transcriptional processing[82,83]. Dysregulation of H19, HOTAIR and MALAT1 was observed in many kind of cancers. Their upregulation was associated with tumor cell proliferation, invasion and metastasis suggesting that these lncRNAs may be potential prognostic biomarkers. Indeed, studies *in vitro* showed that their knockdown could inhibit invasions and metastasis. Therefore the identification of lncRNAs as new biomarkers for clinical use could represent an important finding in the context of rare tumors as GISTs. Although the lncRNAs have already attracted the attention of the scientific community[77], however the clinical significance of the lncRNA expression has been not yet understood. In this study, for the first time, we found that both lncRNAs H19 and MALAT1 were upregulated in tumor specimens of patients with GIST and that the up-regulation of both lncRNAs H19 and MALAT1 was significantly associated with the KIT-mutation status. Indeed the percentage of H19 upregulation was significantly higher in mutated vs wild-type patients, while the percentage of MALAT1 upregulation was lower in mutated vs wild-type patients. According to the known negative prognostic role of c-KIT mutations in GIST, these data would suggest a potential, opposite prognostic value of both H19 and MALT1 lncRNAs in these patients. This suggestion has been confirmed by the analysis of the TTP in the subgroup of patients with advanced disease who received first-line therapy with

imatinib. Indeed the percentage of H19 upregulation was significantly higher in patients with TTP < 3 months, suggesting a negative prognostic role of H19, which seems to be associated with an early PD to imatinib. By contrast, the percentage of upregulation of MALAT1 was significantly higher in patients with TTP > 3 months, suggesting a positive prognostic role of MALAT1, which seems to predict a longer treatment efficacy. Also, we demonstrated a significant lower median TTP in patients with H19 up-regulation as well as a significantly higher median TTP in patients with MALAT1 upregulation vs those with H19 and MALAT1 down-regulation, respectively.

According to these evidences the evaluation of lncRNAs expression could allow to select among all c-KIT mutated GIST patients eligible to receive first-line therapy with imatinib those patients who could really benefit from this treatment reserving a more effective therapy to the others, with interesting implications for their clinical managment.

However the low number of patients included in the study limits of course the scientific validity of our results, which need to be explored and confirmed in prospective studies including larger patients' cohort.

Furthermore the determination of H19, MALAT1 and HOTAIR could has been partially influenced by the following factors: FFPE tissues stability, formalin-fixation and paraffin-embedding may have influenced the stability of lncRNAs; heterogeneity of included population and their clinicalpathological parameters; heterogeneity of information and low amount of lncRNA expression levels to correlate with the clinicalpathological characteristics. Determination principle was the same, analysis type was qRT- PCR. As regards the lncRNA HOTAIR, it has a longer amplicon (152 bp) than H19 and MALAT1. Analysis with bioanalyzer, a software tool designed to help scientists in estimating the integrity of total RNA samples, have shown a RNA Integrity Number < 7, because of the poor quality of RNA, extracted from paraffin tissue. The results of HOTAIR expression levels were indeterminated in all analyzed tumor samples. Through qRT-PCR method, the probe couldn't bind, amplify and express its target, probably because HOTAIR has been degraded during its isolation. By contrast, we tried to analyze the HOTAIR expression levels trough digital real time PCR. We have compared a FFPE GIST sample to its healthy counterpart, using H19 expression level as control. Although we have confirmed the expression of lncRNA H19, obtained

by qRT-PCR, we have not detected any expression of HOTAIR with this third generation method.

Our results indicate that upregulation of H19 and MALAT1 could contribute to oncogenesis of GIST suggesting their potential role as prognostic/predictive biomarkers for clinical use.

Even if very interesting, these preliminary observations need to be confirmed by subsequent larger prospective studies. Of course it will be necessary to expand the study population, establishing collaborations with other centers of reference for rare tumors, to fine tuning the analysis of expression levels through third generation machineries and to confirm our data in fresh tissue. Recent evidences identified lncRNAs in plasma exosome or also in complex with circulating microRNAs. Although these studies clearly demonstrate that there are many functional circulating lncRNA, key questions remain to be solved[84,85]. As recently shown in other tumor types, it would be also interesting evaluating the expression levels of circulating lncRNAs in plasma of GIST patients and comparing them with the results obtained in tumor tissue. In the last years, groups of reasercher have walked roads to identify biomarkers which could help the early detection and screening, the choice of surgical or medical treatments, and the monitoring during the follow- up period. This represents a small but interesting contribute to this research which aims to further personalize the management and treatment of patients with GIST.

CHAPTER 5

Figures and Tables

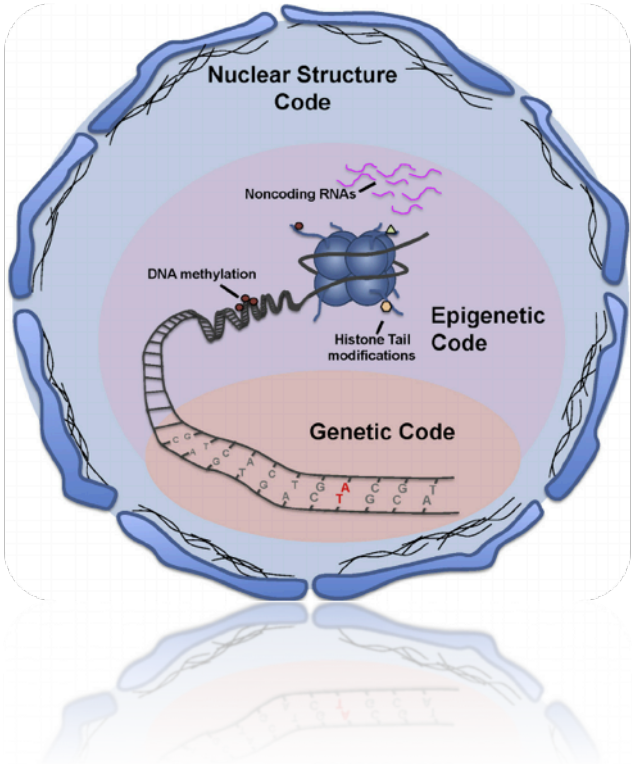

Figure 1| The triple-code hypothesis. The triple-code model includes genetic, epigenetic and nuclear architecture and it determines the functional biological identity of each cell. The genetic code respects Mendel's laws of heredity, but a wrong letter of a gene's nucleotides or other genetic mutation can encodes an aberrant protein. Finally, the epigenetic code is the combination of changes in epigenetic marks (DNA methylation, histone tail modifications) that occur on the nucleosome structure. Moreover, the action of noncoding RNAs molecules contributes to epigenetic phenotype. Image reproduced by Lomberk GA et al., 2015, Surg Clin North Am.[3]

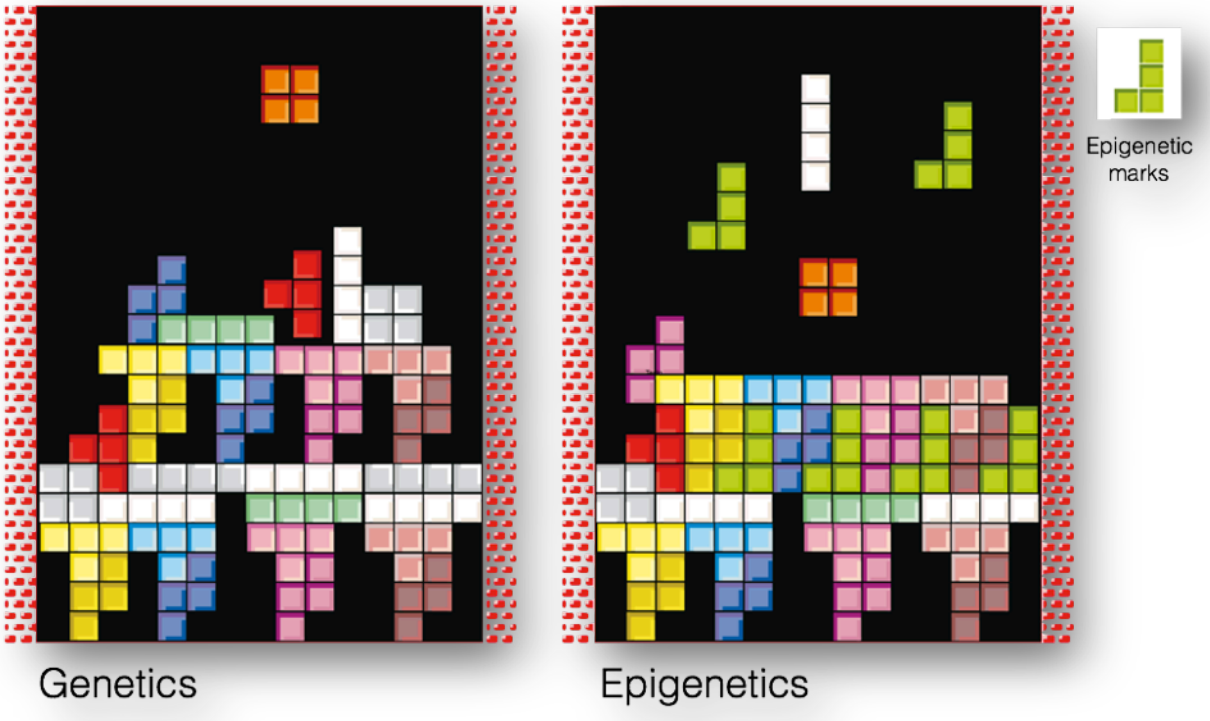

Figure 2| Gen-epigenetic tetris model. Each cell accumulates genetic and epigenetic events that may or may not cause the aberrant state. As in Tetris game, the human organism is planned to correct the genetic errors. If these are not repaired, they may cause the tumor phenotype (left). Instead, the epigenetic events occur physiologically. They tears down the wall of incorrect combination because they are essential for our correct development (right vs left). Otherwise, the tumor phenopype is a resultant of genetics and epigenetics mechanisms. The latter can participate with the arrival of epigenetic marks which can contribute to the development and tumor progression without necessarily notch the gene sequence (right).

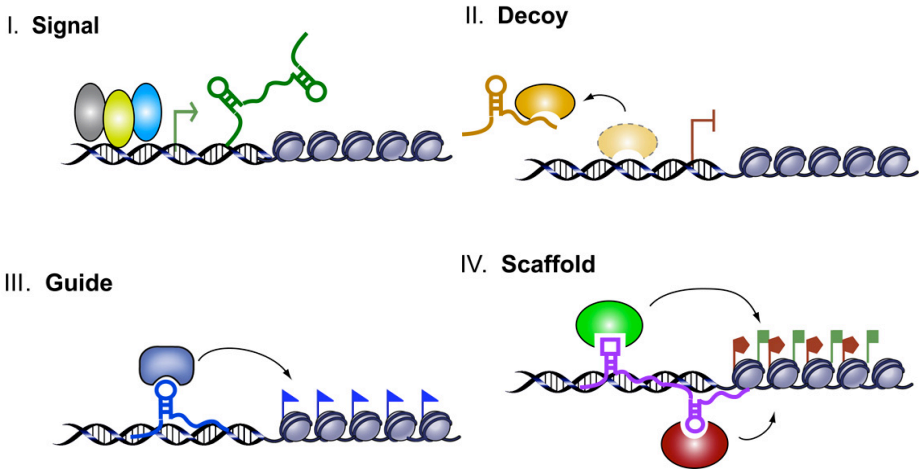

Figure 3| Schematic cellular functions of lncRNAs. I (signal): in a specific space and time, lncRNAs can regulate gene expression recruiting chromatin-modifying factors (colored ovals) or participating in a molecular pathway; II (decoy): lncRNAs can tear off proteins from chromatin, into nuclear subdomains or cytoplasm; III (guide): lncRNAs can recruit, *in cis* or *in trans*, RBP complexes or chromatin modifying enzymes to target genes; IV (scaffold): lncRNAs can form ribonucleoprotein complexes. Image reproduced by Wang et al., 2011, Mol Cell.[21]

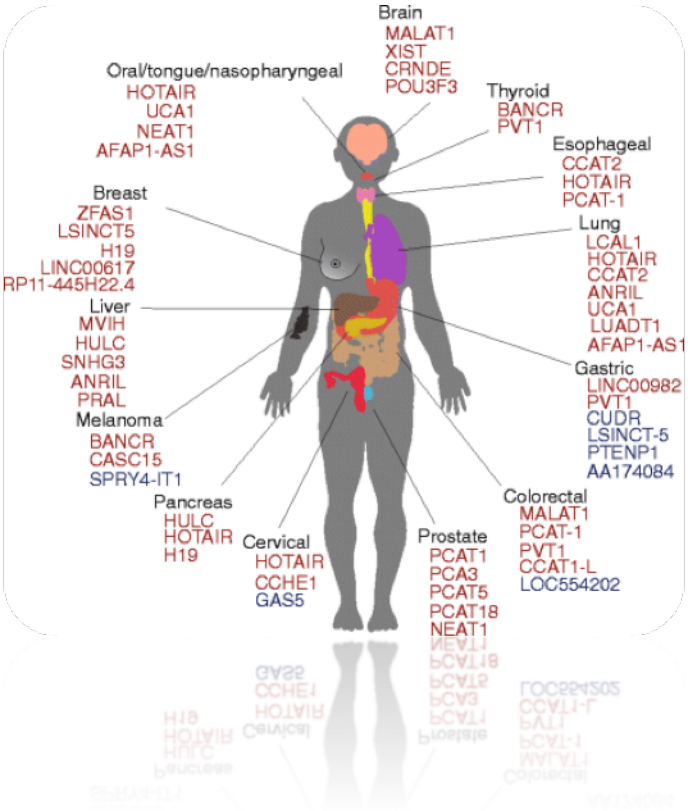

Figure 4|LncRNAs in a different tumoral hystotypes. Even before next generation sequencing, RNA-seq. and successive overlap of sequencing lncRNAs libraries have shown a large number of lncRNAs differentially expressed, both up (red) and down (blue), in different tumoral hystotypes if compared with normal tissues[1,2]. (Image riproduced by Bartonicek N. et al., 2016, Mol Cancer

Table1| Clinical features of the GIST samples

Sex	Male		26
	Female		14
Median age, years			59
Mitotic rate	<5/50 HPF:		10
	≥5/50 HPF		12
Tumor size	<5 cm		14
	≥5 cm		9
Tumor site	Gastro-intestinal		24
	Colon-rectal		2
Risk classification (Miettinen's criteria)	Low risk-very low risk		8
	Intermediate-high risk		8
Analysis mutation	Wild type		13
	Mutated	cKIT	25
		PDGFR	2
Onset	Localized		25
	Metatstatic		15
Imatinib 400/800 mg	Mutated cKIT	Ajuvant	8
		1st line	10

Table2| Primers used for analysis of KIT and PDGFRα genetic aberrations

Exon	Primer Set
9	F:5'-AGC CAG GGC TTT TGT TTT CT-3' R:5'-CAG AGC CTA AAC ATC CCC TTA-3'
11	F:5'-CCT TTG CTG ATT GGT TTC GT-3' R:5'-ACC CAA AAA GGT GAC ATG GA-3'
13	F:5'-GTT CCT GTA TGG TAC TGC ATG CG-3' R:5'-CAG TTT ATA ATC TAG CAT TGC C-3'
17	F:5'-CTG AAT ACT TTA AAA CAA AAG TAT TGG-3' R:5'-TTA TGA AAA TCA CAG GAA ACA ATT T-3'
12	F:5'-AAG CTC TGG TGC ACT GGG ACT T -3'
	R:5'-ATT GTA AAG TTG TGT GCA AGG GA -3'
14	F:5'-CAG GAT TAG TCA TAT TCT TGG TTT TT -3'
	R:5'-TTC TAT TCC CTG CCA TGT GT -3'
18	F:5'-TAC AGA TGG CTT GAT CCT GAG T -3'
	R:5'-AGT GTG GGA GGA TGA GCC TG -3'

Table3| up, down, undetermined lncRNA H19, MALAT1 and HOTAIR in GIST patients and mean of their relative expression values.

lncRNA-ID	Up regulated n (%)	mean log_2(fold change)	Down regulated	Mean log_2(fold change)	Undetermined n (%)
H19	20 (50%)	5	14 (35%)	-3	6 (15%)
MALAT1	14 (45,15%)	4	10 (32,25%)	-0,65	7 (22,6%)
HOTAIR	N.A	N.A	N.A	N.A	40 (100%)

Table 3: N.A.: not available

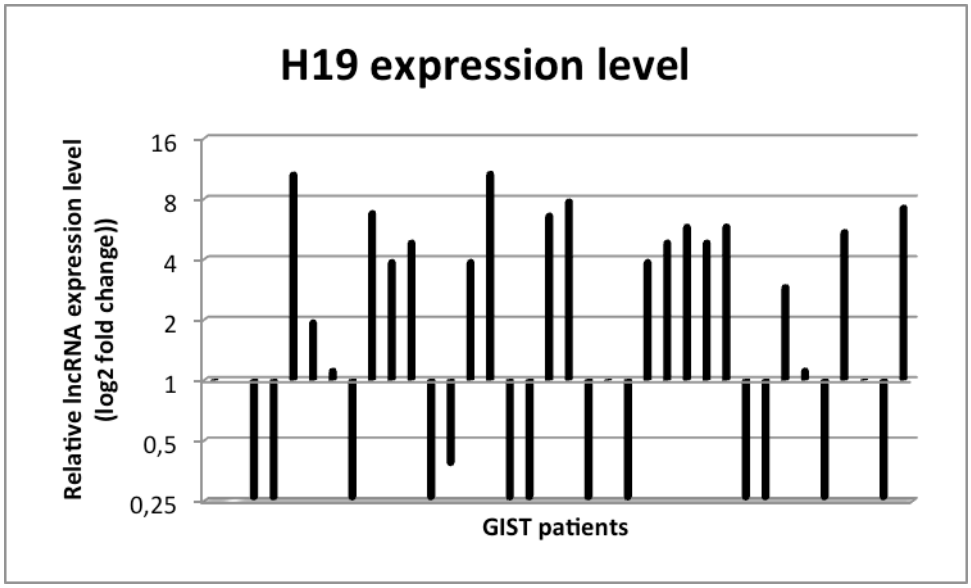

Figure 5| H19 expression levels ($log_2\ 2^{-\Delta\Delta CT}$) in GIST groups. Relative expression levels are represented for each group. Each sample was normalized using the corresponding normal counterpart.

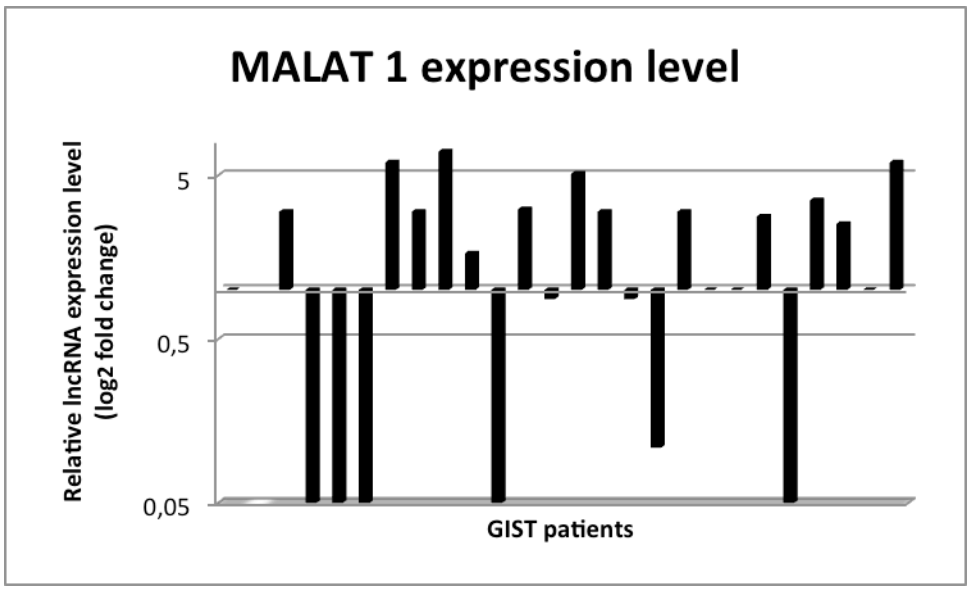

Figure 6| MALAT1 expression levels ($log_2\ 2^{-\Delta\Delta CT}$) in GIST groups. Relative expression levels are represented for each group. Each sample was normalized using the corresponding normal counterpart.

Table4| Results of the association of clinicalpathological parameters with lncRNA-H19 expression level according to group of study

Variable		H19 expression level	
		>1	<1
Total	34	14 (41%)	20 (59%)
Mitotic rate: n.(%)	<5/50 HPF: 9 (45%) ≥5/50 HPF: (55%)	4 (44,44%) 4 (36,4%)	5 (55,6%) 7 (63,6%)
Tumor size: n.(%)	<5 cm: 12(%) ≥5 cm: 8 (%)	6 (50%) 3 (37,5%)	6 (50%) 5 (62,5%)
Tumor site	Gastro-intestinal: 22 (91,66%) Colon-rectum: 2 (%)	13 (59%) 2 (100%)	9 (41%) 0
Risk classification (Miettinen's criteria)	Low risk-very low risk: 7 (50%) Intermediate-high risk: 7 (50%)	3 (42,9%) 4 (57,1%)	4 (57,1%) 3 (42,9%)
Onset	Localized: 24 (%) Metastatic: 10(%)	14 (%) 6 (%)	10 (%) 4 (%)
Analysis mutation	Wild type.: 7 (23,3%) Mutated.: 23 (76,7%)	4(57,1%) 17 (74%)	3 (42,9%) 6 (26%)
TTP:	Early (< 3 months): 5 (50%) Late (> 3 months): 5 (50%)	5 (100%) 1 (20%)	0 4 (80%)
TTP: median (weeks)		7,5	22,5

Table 5| Results of the association of clinicalpathological parameters with lncRNA-MALAT1 expression level according to group of study

Variable		MALAT1 expression level	
		>1	<1
Total	24	10 (41,6%)	14 (58,4%)
Mitotic rate	<5/50 HPF: 5 (38,5%) ≥5/50 HPF: 8 (61,5%)	3 (60%) 5 (63,5%)	2 (40%) 3 (37,5%)
Tumor size	<5 cm: 7(58,4%%) ≥5 cm: 5 (41,6%)	6 (85,7%) 3 (60%)	1 (14,3%) 2 (40%)
Tumor site	Gastro-intestinal: 13(%) Colon-rectum: 2 (%)	7 (%) 2 (100%)	6 (%) 0
Risk classification (Miettinen's criteria)	Low risk-very low risk: 4(50%) Intermediate-high risk: 4(50%)	2 (50%) 3 (75%)	2 (50%) 1 (25%)
Onset	Localized: 24 (60,6%) Metastatic: 10 (29,4%)	13 (54,2%) 5 (50%)	11 (45,8%) 5 (50%)
Analysis mutation	Wild type.: 4 (19%) Mutated.: 17 (80,9%)	4 (100%) 13 (76,5%)	- 4 (23,5%)
TTPI	Early (< 3 months): 5 (50%) Late (> 3 months): 5 (50%)	1 (20%) 3 (60%)	4 (80%) 2 (40%)
TTP: median (weeks)		15	3

Bibliografy

1. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature.* 2009;458(7235):223-227.
2. Yan X, Hu Z, Feng Y, et al. Comprehensive Genomic Characterization of Long Non-coding RNAs across Human Cancers. *Cancer Cell.* 2015;28(4):529-540.
3. Lomberk GA, Urrutia R. The Triple-Code Model for Pancreatic Cancer: Cross Talk Among Genetics, Epigenetics, and Nuclear Structure. *Surg Clin North Am.* 2015;95(5):935-952.
4. Gerstein MB, Bruce C, Rozowsky JS, et al. What is a gene, post-ENCODE? History and updated definition. *Genome Res.* 2007;17(6):669-681.
5. Triantaphyllopoulos KA, Ikonomopoulos I, Bannister AJ. Epigenetics and inheritance of phenotype variation in livestock. *Epigenetics Chromatin.* 2016;9:31.
6. Nestler EJ. Hidden switches in the mind. *Sci Am.* 2011;305(6):76-83.
7. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene.* 2002;21(35):5427-5440.
8. Mattick JS. The genetic signatures of noncoding RNAs. *PLoS Genet.* 2009;5(4):e1000459.
9. Feil R. Environmental and nutritional effects on the epigenetic regulation of genes. *Mutat Res.* 2006;600(1-2):46-57.
10. Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS. Non-coding RNAs: regulators of disease. *J Pathol.* 2010;220(2):126-139.
11. Okamoto I, Arnaud D, Le Baccon P, et al. Evidence for de novo imprinted X-chromosome inactivation independent of meiotic inactivation in mice. *Nature.* 2005;438(7066):369-373.
12. Gupta RA, Shah N, Wang KC, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature.* 2010;464(7291):1071-1076.
13. Khalil AM, Guttman M, Huarte M, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci U S A.* 2009;106(28):11667-11672.
14. Mirabella AC, Foster BM, Bartke T. Chromatin deregulation in disease. *Chromosoma.* 2016;125(1):75-93.
15. Joyce GF. The antiquity of RNA-based evolution. *Nature.* 2002;418(6894):214-221.
16. Amaral PP, Dinger ME, Mercer TR, Mattick JS. The eukaryotic genome as an RNA machine. *Science.* 2008;319(5871):1787-1789.
17. Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF. RNA regulation of epigenetic processes. *Bioessays.* 2009;31(1):51-59.
18. Nagano T, Fraser P. No-nonsense functions for long noncoding RNAs. *Cell.* 2011;145(2):178-181.
19. Carninci P, Kasukawa T, Katayama S, et al. The transcriptional landscape of the mammalian genome. *Science.* 2005;309(5740):1559-1563.
20. Pennisi E. Shining a light on the genome's 'dark matter'. *Science.* 2010;330(6011):1614.
21. Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. *Mol Cell.* 2011;43(6):904-914.
22. Zhao Y, Li H, Fang S, et al. NONCODE 2016: an informative and valuable data source of long non-coding RNAs. *Nucleic Acids Res.* 2016;44(D1):D203-208.
23. Derrien T, Johnson R, Bussotti G, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res.* 2012;22(9):1775-1789.

24. Bernstein E, Allis CD. RNA meets chromatin. *Genes Dev.* 2005;19(14):1635-1655.
25. Bracken AP, Helin K. Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat Rev Cancer.* 2009;9(11):773-784.
26. Faghihi MA, Wahlestedt C. Regulatory roles of natural antisense transcripts. *Nat Rev Mol Cell Biol.* 2009;10(9):637-643.
27. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet.* 2009;10(3):155-159.
28. Wilusz JE, Sunwoo H, Spector DL. Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev.* 2009;23(13):1494-1504.
29. Dinger ME, Gascoigne DK, Mattick JS. The evolution of RNAs with multiple functions. *Biochimie.* 2011;93(11):2013-2018.
30. Dinger ME, Pang KC, Mercer TR, Mattick JS. Differentiating protein-coding and noncoding RNA: challenges and ambiguities. *PLoS Comput Biol.* 2008;4(11):e1000176.
31. Chen LL, Carmichael GG. Long noncoding RNAs in mammalian cells: what, where, and why? *Wiley Interdiscip Rev RNA.* 2010;1(1):2-21.
32. Angrand PO, Vennin C, Le Bourhis X, Adriaenssens E. The role of long non-coding RNAs in genome formatting and expression. *Front Genet.* 2015;6:165.
33. Xu J, Li CX, Li YS, et al. MiRNA-miRNA synergistic network: construction via co-regulating functional modules and disease miRNA topological features. *Nucleic Acids Res.* 2011;39(3):825-836.
34. Xu J, Li Y, Li X, et al. Dissection of the potential characteristic of miRNA-miRNA functional synergistic regulations. *Mol Biosyst.* 2013;9(2):217-224.
35. Li Y, Chen J, Zhang J, et al. Construction and analysis of lncRNA-lncRNA synergistic networks to reveal clinically relevant lncRNAs in cancer. *Oncotarget.* 2015;6(28):25003-25016.
36. Wang X, Arai S, Song X, et al. Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. *Nature.* 2008;454(7200):126-130.
37. Hu Y, Chen HY, Yu CY, et al. A long non-coding RNA signature to improve prognosis prediction of colorectal cancer. *Oncotarget.* 2014;5(8):2230-2242.
38. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem.* 2012;81:145-166.
39. Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. *RNA Biol.* 2012;9(6):703-719.
40. Fanale D, Barraco N, Listì A, Bazan V, Russo A. Non-coding RNAs Functioning in Colorectal Cancer Stem Cells. *Adv Exp Med Biol.* 2016;937:93-108.
41. Piccolo LL, Corona D, Onorati MC. Emerging roles for hnRNPs in post-transcriptional regulation: what can we learn from flies? *Chromosoma.* 2014;123(6):515-527.
42. Postepska-Igielska A, Giwojna A, Gasri-Plotnitsky L, et al. LncRNA Khps1 Regulates Expression of the Proto-oncogene SPHK1 via Triplex-Mediated Changes in Chromatin Structure. *Mol Cell.* 2015;60(4):626-636.
43. Yoon JH, Abdelmohsen K, Gorospe M. Functional interactions among microRNAs and long noncoding RNAs. *Semin Cell Dev Biol.* 2014;34:9-14.
44. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature.* 2010;465(7301):1033-1038.
45. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature.* 2008;455(7209):64-71.
46. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-233.
47. Bartonicek N, Maag JL, Dinger ME. Long noncoding RNAs in cancer: mechanisms of action and technological advancements. *Mol Cancer.* 2016;15(1):43.
48. Amaral PP, Neyt C, Wilkins SJ, et al. Complex architecture and regulated expression of the Sox2ot locus during vertebrate development. *RNA.* 2009;15(11):2013-2027.

49. Ravasi T, Suzuki H, Pang KC, et al. Experimental validation of the regulated expression of large numbers of non-coding RNAs from the mouse genome. *Genome Res.* 2006;16(1):11-19.
50. Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. *Cancer Discov.* 2011;1(5):391-407.
51. Xu C, Yang M, Tian J, Wang X, Li Z. MALAT-1: a long non-coding RNA and its important 3' end functional motif in colorectal cancer metastasis. *Int J Oncol.* 2011;39(1):169-175.
52. Tabano S, Colapietro P, Cetin I, et al. Epigenetic modulation of the IGF2/H19 imprinted domain in human embryonic and extra-embryonic compartments and its possible role in fetal growth restriction. *Epigenetics.* 2010;5(4):313-324.
53. Lustig-Yariv O, Schulze E, Komitowski D, et al. The expression of the imprinted genes H19 and IGF-2 in choriocarcinoma cell lines. Is H19 a tumor suppressor gene? *Oncogene.* 1997;15(2):169-177.
54. Yang F, Bi J, Xue X, et al. Up-regulated long non-coding RNA H19 contributes to proliferation of gastric cancer cells. *FEBS J.* 2012;279(17):3159-3165.
55. Liu FT, Pan H, Xia GF, Qiu C, Zhu ZM. Prognostic and clinicopathological significance of long noncoding RNA H19 overexpression in human solid tumors: evidence from a meta-analysis. *Oncotarget.* 2016.
56. Arita T, Ichikawa D, Konishi H, et al. Circulating long non-coding RNAs in plasma of patients with gastric cancer. *Anticancer Res.* 2013;33(8):3185-3193.
57. Yoshimizu T, Miroglio A, Ripoche MA, et al. The H19 locus acts in vivo as a tumor suppressor. *Proc Natl Acad Sci U S A.* 2008;105(34):12417-12422.
58. Hao Y, Crenshaw T, Moulton T, Newcomb E, Tycko B. Tumour-suppressor activity of H19 RNA. *Nature.* 1993;365(6448):764-767.
59. Li H, Yu B, Li J, et al. Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. *Oncotarget.* 2014;5(8):2318-2329.
60. Cai X, Cullen BR. The imprinted H19 noncoding RNA is a primary microRNA precursor. *RNA.* 2007;13(3):313-316.
61. Liu G, Xiang T, Wu QF, Wang WX. Long Noncoding RNA H19-Derived miR-675 Enhances Proliferation and Invasion via RUNX1 in Gastric Cancer Cells. *Oncol Res.* 2016;23(3):99-107.
62. Zhuang M, Gao W, Xu J, Wang P, Shu Y. The long non-coding RNA H19-derived miR-675 modulates human gastric cancer cell proliferation by targeting tumor suppressor RUNX1. *Biochem Biophys Res Commun.* 2014;448(3):315-322.
63. Matouk IJ, Raveh E, Abu-lail R, et al. Oncofetal H19 RNA promotes tumor metastasis. *Biochim Biophys Acta.* 2014;1843(7):1414-1426.
64. Rinn JL, Kertesz M, Wang JK, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell.* 2007;129(7):1311-1323.
65. Shi Y, Lan F, Matson C, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell.* 2004;119(7):941-953.
66. Yao Y, Li J, Wang L. Large intervening non-coding RNA HOTAIR is an indicator of poor prognosis and a therapeutic target in human cancers. *Int J Mol Sci.* 2014;15(10):18985-18999.
67. Hutchinson JN, Ensminger AW, Clemson CM, Lynch CR, Lawrence JB, Chess A. A screen for nuclear transcripts identifies two linked noncoding RNAs associated with SC35 splicing domains. *BMC Genomics.* 2007;8:39.
68. Clemson CM, Hutchinson JN, Sara SA, et al. An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. *Mol Cell.* 2009;33(6):717-726.
69. Bernard D, Prasanth KV, Tripathi V, et al. A long nuclear-retained non-coding RNA regulates synaptogenesis by modulating gene expression. *EMBO J.* 2010;29(18):3082-3093.

70. Wilusz JE, Freier SM, Spector DL. 3' end processing of a long nuclear-retained noncoding RNA yields a tRNA-like cytoplasmic RNA. *Cell.* 2008;135(5):919-932.
71. Sültmann H, Diederichs S. Long noncoding RNA: "LNCs" to cancer. *Eur Urol.* 2014;65(6):1152-1153.
72. Weber DG, Johnen G, Casjens S, et al. Evaluation of long noncoding RNA MALAT1 as a candidate blood-based biomarker for the diagnosis of non-small cell lung cancer. *BMC Res Notes.* 2013;6:518.
73. Ji P, Diederichs S, Wang W, et al. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene.* 2003;22(39):8031-8041.
74. Wang Y, Xue D, Li Y, et al. The Long Noncoding RNA MALAT-1 is A Novel Biomarker in Various Cancers: A Meta-analysis Based on the GEO Database and Literature. *J Cancer.* 2016;7(8):991-1001.
75. Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? *Oncogene.* 2012;31(43):4577-4587.
76. Niinuma T, Suzuki H, Nojima M, et al. Upregulation of miR-196a and HOTAIR drive malignant character in gastrointestinal stromal tumors. *Cancer Res.* 2012;72(5):1126-1136.
77. Lee NK, Lee JH, Kim WK, et al. Promoter methylation of PCDH10 by HOTAIR regulates the progression of gastrointestinal stromal tumors. *Oncotarget.* 2016.
78. Miettinen M, Lasota J. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Arch Pathol Lab Med.* 2006;130(10):1466-1478.
79. Miettinen M, El-Rifai W, H L Sobin L, Lasota J. Evaluation of malignancy and prognosis of gastrointestinal stromal tumors: a review. *Hum Pathol.* 2002;33(5):478-483.
80. Zhao X, Yue C. Gastrointestinal stromal tumor. *J Gastrointest Oncol.* 2012;3(3):189-208.
81. Chen X, Yan CC, Zhang X, You ZH. Long non-coding RNAs and complex diseases: from experimental results to computational models. *Brief Bioinform.* 2016.
82. Yu B, Shan G. Functions of long noncoding RNAs in the nucleus. *Nucleus.* 2016;7(2):155-166.
83. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell.* 2009;136(4):629-641.
84. Qi P, Zhou XY, Du X. Circulating long non-coding RNAs in cancer: current status and future perspectives. *Mol Cancer.* 2016;15(1):39.
85. Fayda M, Isin M, Tambas M, et al. Do circulating long non-coding RNAs (lncRNAs) (LincRNA-p21, GAS 5, HOTAIR) predict the treatment response in patients with head and neck cancer treated with chemoradiotherapy? *Tumour Biol.* 2016;37(3):3969-3978.

In extenso:

- Oncotarget. 2016 Dec 31. doi: 10.18632/oncotarget.14409. [Epub ahead of print]

"Back to a false normality": new intriguing mechanisms of resistance to PARP inhibitors.

Incorvaia L1, Passiglia F1, Rizzo S1, Galvano A1, Listì A1, Barraco N1, Maragliano R1, Calò V1, Natoli C2, Ciaccio M3, Bazan V1, Russo A1.
1Department of Surgical, Oncological and Oral Sciences, University of Palermo, Palermo, Italy.
2Department of Medical, Oral and Biotechnological Sciences, Centre of Ageing Sciences and Translational Medicine - CESI-MeT University "G. D'Annunzio", Chieti, Italy.
3Section of Clinical Biochemistry and Clinical Molecular Medicine, Department of Biopathology and Medical Biotechnology, University of Palermo - U.O.C. Laboratory Medicine - CoreLab, Policlinico University Hospital, Palermo, Italy.

- Adv Exp Med Biol. 2016;937:93-108. doi: 10.1007/978-3-319-42059-2_5.

Non-coding RNAs Functioning in Colorectal Cancer Stem Cells.

Fanale D1, Barraco N1, Listì A1, Bazan V1, Russo A2.
1Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy.
2Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy. antonio.russo@usa.net.

- Ther Adv Med Oncol. 2016 May;8(3):188-97. doi: 10.1177/1758834016630976. Epub 2016 Feb 16.

Nintedanib in NSCLC: evidence to date and place in therapy.

Bronte G1, Passiglia F1, Galvano A1, Barraco N1, Listì A1, Castiglia M1, Rizzo S1, Fiorentino E1, Bazan V1, Russo A2.
1Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Italy.
2Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy.

- Oncotarget. 2016 May 17;7(20):29321-32. doi: 10.18632/oncotarget.8722.

Can the microRNA expression profile help to identify novel targets for zoledronic acid in breast cancer?

Fanale D1, Amodeo V1, Bazan V1, Insalaco L1, Incorvaia L1, Barraco N1, Castiglia M1, Rizzo S1, Santini D2, Giordano A3, Castorina S4,5, Russo A1.
1Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Palermo, Italy.
2University Campus Bio-Medico, Department of Medical Oncology, Rome, Italy.
3Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, PA, USA.
4Fondazione Mediterranea "G.B. Morgagni", Catania, Italy.
5Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy.

- Tumour Biol. 2016 Jun;37(6):7109-18. doi: 10.1007/s13277-016-5044-8. Epub 2016 Apr 4.

Dietary restriction: could it be considered as speed bump on tumor progression road?

Cangemi A1, Fanale D1, Rinaldi G1, Bazan V1, Galvano A1, Perez A1, Barraco N1, Massihnia D1, Castiglia M1, Vieni S1, Bronte G1, Mirisola M1, Russo A2.
1Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via

del Vespro 129, 90127, Palermo, Italy.
2Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy. antonio.russo@usa.net.

- Tumour Biol. 2016 Apr;37(4):4263-73. doi: 10.1007/s13277-016-4856-x. Epub 2016 Jan 20.

A headlight on liquid biopsies: a challenging tool for breast cancer management.

Massihnia D1, Perez A1, Bazan V1, Bronte G1, Castiglia M1, Fanale D1, Barraco N1, Cangemi A1, Di Piazza F1, Calò V1, Rizzo S1, Cicero G1, Pantuso G1, Russo A2.
1Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy.
2Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy. antonio.russo@usa.net.

- Anal Cell Pathol (Amst). 2015;2015:690916. doi: 10.1155/2015/690916. Epub 2015 Sep 21.

Stabilizing versus destabilizing the microtubules: a double-edge sword for an effective cancer treatment option?

Fanale D1, Bronte G1, Passiglia F1, Calò V1, Castiglia M1, Di Piazza F1, Barraco N1, Cangemi A1, Catarella MT1, Insalaco L1, Listì A1, Maragliano R1, Massihnia D1, Perez A1, Toia F2, Cicero G1, Bazan V1.
1Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, 90127 Palermo, Italy.
2Department of Surgical, Oncological and Oral Sciences, Section of Plastic Surgery, University of Palermo, 90127 Palermo, Italy.

Book:

- Advances in Experimental Medicine and Biology Volume 937 OndrejSlaby • GeorgeA.Calin Editors

Non-coding RNAs in Colorectal Cancer Chapter 5: **Non-coding RNAs Functioning in Colorectal Cancer Stem Cells.**

Fanale D[1], Barraco N[1], Listì A[1], Bazan V[1], Russo A[2].
[1]Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy.
[2]Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy.

Abstract:

- Annals of Oncology 27, 2016

The role of microRNAs in driving EGFR-TKI resistance in NSCLC cell lines

A. Perez1, M. Castiglia2, F. Passiglia2, N. Barraco2, A. Cangemi2, D. Fanale2, A. Listì2, R. Maragliano2, D. Massihnia, F. Di Piazza2, S. Vieni2, V. Calò2, S. Rizzo2, L. Incorvaia2, V. Bazan2, A. Russo2
1AOUP "Paolo Giaccone", Palermo
1AOUP "Paolo Giaccone", DICHIRONS Palermo

- Annals of Oncology 27, 2016

The prognostic role of KRAS and BRAF in patients undergoing surgical resection of colorectal cancer liver metastasis: a systemic review and meta-analysis

F. Passiglia, A. Galvano, S. Rizzo, A. Listì, N. Barraco, R. Maragliano, L. Insalaco, E. Bronte, I. Alessi, A. Guarini, L. Terruso, L. Castellana, A. Perez, D. Massihnia F. Di Piazza, V. Calò, M. Castiglia, V. Bazan, A. Russo

AOUP "Paolo Giaccone", Palermo

- GOIM 18-20 Giugno /2016

SICILIAN PATIENTS WITH familial MALIGNANT melanoma SHOW A very low frequency of germline CDKN2A mutations.

Rinaldi G.[1], Di Lorenzo S.[2], Fanale D.[1], Calò V.[1], Corradino B.[2], Barraco N.[1], Cangemi A.[1], Castiglia M.[1], Di Piazza F.[1], Di Stefano B.[1], Incorvaia L.[1], Listì A.[1], Maragliano R.[1], Massihnia D.[1], Passiglia F.[1], Perez A.[1], Viviana Bazan[1], Adriana Cordova[2], Antonio Russo[1].

[1] Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, 90127 Palermo, Italy;

[2] Department of Surgical, Oncological and Oral Sciences, Section of Plastic Surgery, University of Palermo, 90127 Palermo, Italy

- GOIM 18-20 Giugno /2016

MiRNAs in metaplastic transformation of the esophagus: something in the way toward carcinogenesis

Cabibi D[1], Caruso S.[2], Fanale D.[2], Ingrao S.[1], Barraco N.[2], Calò V.[2], Cangemi A.[2], Castiglia M.[2], Di Piazza F.[2], Di Stefano B.[2], Galvano A.[2], Incorvaia L.[2], Listì A.[2], Maragliano R.[2], Massihnia D.[2], Perez A.[2], Passiglia F.[2], Pantuso G.[3], Fiorentino E.[3], Bazan V.[2], Russo A.[2]

[1] Department of Science for Promotion of Health and Mother and Child Care, Section of Human Pathology, University of Palermo, 90127 Palermo, Italy;

[2] Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, 90127 Palermo, Italy;

[3] Department of Surgical, Oncological and Oral Sciences, Section of Surgical Oncology, University of Palermo, 90127 Palermo, Italy

- GOIM 18-20 Giugno /2016

Potential miRNAs involved in molecular pathways mediating the anticancer effects of zoledronic acid in breast cancer cells

Fanale D., Amodeo V., Insalaco L., Barraco N., Calò V., Cangemi A., Castiglia M., Di Piazza F., Di Stefano B., Incorvaia L., Listì A., Maragliano R., Massihnia D., Perez A., Bazan V. and Russo A.

Department of Surgical, Oncological and Oral Sciences, Section of Medical Oncology, University of Palermo, 90127 Palermo, Italy.

- ASCO Abstract 3565 - 2016 ASCO Annual Meeting (June 4 - 6, 2016)

KRAS and BRAF as prognostic biomarkers in patients undergoing surgical resection of colorectal cancer liver metastasis: a systematic review and meta-analysis

Francesco Passiglia1, Giuseppe Bronte1, Sergio Rizzo1, Antonio Galvano1, Angela Listì1, Nadia Barraco1, Marta Castiglia1, Valentina Calò1, Viviana Bazan1, Giuseppe Cicero1, Christian Rolfo2, Antonio Russo1.

1Medical Oncology Unit, Department of Surgical, Oncological and Oral Sciences, University of Palermo, Palermo, Italy

- Annals of Oncology 26, 2015http://annonc.oxfordjournals.org/content/26/suppl_6/vi29.3

Into the Wild of long non-coding RNAs in Gastrointestinal Stromal Tumors (GISTs) to explore new prognostic/predictive biomarkers

Barraco Nadia[1]; Listì Angela; Maragliano Rossella; Bazan Viviana; Badalamenti Giuseppe; Fulfaro Fabio; Incorvaia Lorena; Calò Valentina; Castiglia Marta; Bronte Giuseppe; Cangemi Antonina; Perez Alessandro; Insalaco Lavinia; Bronte Enrico; Russo Antonio.

[1] Medical Oncology Unit, Departmente of Surgical, Oncological and Oral Sciences, University of Palermo, Palermo, Italy.

- Annals of Oncology 26, 2015

Study of mutational status of Sicilian GISTs patients

M. Castiglia, G. Badalamenti, F. Fulfaro, L. Incorvaia, V. Calò, V. Bazan, N. Barraco, D. Massihnia, G. Bronte, A. Russo

Policlinico Universitario “Paolo Giaccone”, Palermo

- **IASLC** (September 9, 2015)

PD-L1 expression as predictive biomarker in patients with NSCLC: a pooled analysis

Francesco Passiglia[1], Giuseppe Bronte[1], Sergio Rizzo[1], Antonio Galvano[1], Giovanni Sortino[1], Emmanuela Musso[1], Angela Listì[1], Nadia Barraco[1], Marta Castiglia[1], Valentina Calò[1], Viviana Bazan[1], Giuseppe Cicero[1], Christian Rolfo[2], Antonio Russo[1].

[1]Medical Oncology Unit, Department of Surgical, Oncological and Oral Sciences, University of Palermo, Palermo, Italy

[2]Phase I- Early Clinical Trials Unit, Oncology Department and Multidisciplinary Oncology Center Antwerp (MOCA), Antwerp University Hospital, Edegem, Belgium

“Bisogna sempre credere alle nostre osservazioni, per quanto bizzarre possano essere.

forse stanno cercando di dirci qualcosa” (Barbara McClintock)