World Cancer Research Journal WCRJ 2014; 1 (4): e360

PHARMACOGENOMICS OF GEMCITABINE IN THE TAILOR-MADE THERAPY

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ABSTRACT: Backbone: *Gemcitabine is an anticancer drug routinely used to treat diverse cancers disease. Discontinuation of Gemcitabine based treatments is mostly caused either by toxicity or more often for tumor progression, potentially compromising patient benefit. Several strategies to prevent toxicity/resistance have been so far investigated.*

Content: *In order to overreach this life-conditioning side effect, we describe in detail recent findings about the underlying mechanism of genetic variants associated with toxicity and resistance to Gemcitabine-based chemotherapy. A comprehensive panel of 6 polymorphisms detected on 4 genes, previously validated as significant markers related to toxicity/resistance, arer proposed and finely described. In addition, an early outline evaluation of the genotyping costs and methods are taken in consideration.*

Summary: *Based on the individual pharmacogenomics profile, the oncologists will have new means to make treatment decisions for their patients in order to maximize benefit and minimize toxicity. Based on this purpose, the clinician and lab manager should cooperate to evaluate advantages and limitations, in terms of costs and applicability, of the most appropriate pharmacogenomics tests to incorporate routinely into clinical practice.*

KEY WORDS: *Pharmacogenomics, Gemcitabine, Cytarabine, Genotyping costs, Cytidine Deaminase, DNA Repair genes.*

INTRODUCTION

In clinical practice, Pharmacogenomics and Pharmacogenetics (PGx) testing may enable clinicians to identify patients who are less likely to benefit from expensive therapies, those who are susceptible to severe treatment-related toxicities/resistance at standard doses, reducing delays for the patient, and to receive perhaps more tailored alternative treatments. The potential promises in the field of PGx are certainly attractive for anti-cancer drugs because many chemotherapeutic agents have a limited treatment index and often cause acute life threatening toxicities[1].

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Gemcitabine is the backbone (both induction and consolidation) of a multitude of regimens for the treatments of diverse neoplasms like lung[2], head and neck[3], uro-gynecological[4], pancreatic carcinomas[5] and lymphoma[6] .

Toxicity profile of Gemcitabine is well documented when given at standard or high doses; it is often responsible for the suspension of therapy and for the impairment of the patient's benefit. Primarily toxicities include severe bone marrow depression[7]. Despite its clinical benefit in frontline treatment of AML (achieving 65-80% of complete response), many patients shown suboptimal clinical outcome and relapse as consequence to cellular Gemcitabine resistance phenomena[8].

It is known that, Gemcitabine and others antimetabolite drugs undergo complex metabolic biotransformation, prior incorporation in *de novo* DNA. Gene products (enzymes) involved in this process have been well documented[9]. They include: Cytidine Deaminase (CDA); human Equilibrative Nucleoside Transporters (hENTs) and some DNA-repair genes.

Some adverse drug response in cytarabine-based therapy could be elucidated by diverse single nucleotide polymorphisms (SNPs) found on the citied genes[8].

However, if the detection of these genetic variants on previously citied genes is routinely incorporated either into clinical practice or into large clinical trials, knowledge concerning the predictive value of PGx which will eventually enable the individualization of optimized therapy will be gained. However, it is still necessary a precise demonstration that PGx tests offer an added value, in terms of relative cost and benefit. Furthermore, trials evaluating the pharmacoeconomic impact of genotyping testing in Gemcitabine-based therapy will likely provide answers for policy integration of PGx testing in clinical practice. Overviews of cost-effectiveness studies on PGx technologies are now available[10, 11]. A relevant example is provided by the National Institute for Health and Clinical Excellence (NICE). NICE forms a Diagnostic Advisory committee willing to stimulate Pharma and Academic communities to produce a comprehensive set of data, including design and data sources in economic models of healthcare[12].

The aim of this review is to provide information for the oncologist on the advantages and limitations of the most common available PGx testing for prevention of toxicity/resistance through molecular evaluation of a panel of 6 SNPs, detectable on 4 genes involved in Gemcitabine biotransformation.

Equally, PGx test would also be applicable to prevent toxicity/resistance to other nucleoside analogs which are metabolized by the same pathway, as Cytarabine[13], Fludarabine[14], Decitabine and so on[15].

Hopefully, oncologists will have new means based on the individual genetic profile of their patients to tail treatment decisions in order to minimize toxicity and avoid resistance to Gemcitabine based-therapy.

GENOTYPING PANEL ASSAY

Several criteria are used to select polymorphisms for pharmacogenomics panel tests (Table 1 and 2):

a) Searching the most validated genetic variants known to influence the Pharmacokinetics/pharmacodynamics of Gemcitabine (www.pharmagkb.org);
b) Reviewing the most recent studies upgrading in clinical research, in particular, trials including polymorphisms related to toxicity/resistance (www.clinicaltrials.gov); a review of all the literature was consulted and all the pertinent articles were manually searched and analyzed matching concordant key words: “Gemcitabine” [MeSH] and “Pharmacogenomics” [MeSH], limited to, therapy AND toxicity, limited to human subjects and the English language (www.ncbi.nlm.nih.gov/sites/entrez).
c) Issues evaluating the pharmacoeconomic impact of genotyping testing, likely providing answers for policy making in the incorporation of PGx markers into clinical practice.

However, the fine molecular function of these SNPs remains unclear and controversial. Furthermore, there are many genes whose effects on toxicity to Gemcitabine have yet to be extensively studied.

GENES AND VARIANTS IN THE METABOLIC PATHWAY OF GEMCITABINE

Generally, two approaches are applied to identify genes and their variation implicated in the metabolic pathway of NTs. The first one is able to interrogate a large number of genes involved in a specific pathway conventionally named Genomic Wide Association Studies (GWAS). It is based on large scale of genotyping platforms on “in vitro” studies. Most of the putative markers associated with the dNTs efficacy have emerged from Candidate-Gene Studies (CGS) approach. As much attention has focused on the genes and SNPs related

TABLE 1. CDA HAPLOTYPE PANEL

	CDA haplotypes**								
Nucleotide position	***1A**	***1B**	***1C**	***1D**	***1L**	***1Q**	***2A**	***2B**	***2D**
-451C>T	C	C	C	C	**T**	C	**T**	**T**	C
-92A>G	A	A	A	A	**G**	**G**	**G**	**G**	A
-31Ins/Del	Ins	**Del**	**Del**	I	**Del**	**D**	**D**	**D**	I
79AC>T	A	A	A	A	A	A	**C**	**C**	**C**
435C>T	C	C	T	T	C	C	**T**	C	**T**
Frequency	0.47	0.03	0.14	0.02	0.01	0.01	0.13	0.21	0.01

**haplotype nomenclature taken from Gilbert JA et al. Clin Cancer Res 2006;12:1794-803.
In bold are reported minor allele polymorphisms.

to mechanisms of drug transport, activation, degradation and DNA repair.

Broadly, drug transporters are classified in two major families, named "uptake transporters" of Solute carrier (SLC) family, and "Efflux Transporters" of the ATP-binding Cassette (ABC) family (http://nutrigene.4t.com/humanabc.htm).

Gemcitabine and othe nucleoside analogs are typically hydrophilic and require (at standard dose) nucleoside transporter proteins to uptake efficiently inside the cells. There is a substantial clinical evidence indicating that the efficiency of intracellular Gemcitabine concentration mediated by Nucleoside transporters (NTs) is related to clinical outcome[16].

Several membrane-bound NTs protein family are identified in humans like as Anion Organic Transporters (OATs alias SLC22), Concentrative Nucleside Transporters (hCNT alias SLC 28) and Equilibrative Nucleoside Transporter (hENT alias SLC29). In particular, Gemcitabine are preferentially permeant for hENT1, hCNT1 and hCNT3.

hENT1 (SLC29A1) appears to be highly conserved and no functional variants in the coding region have been significantly identified[17]. In fact, differential expression of NTs across plasma membrane among neoplastic tissues and individuals phenotype is produced by the presences of genetic variants in promoter region (5'UTR) of the hENT1. Study[18] on the structure and function of **hCNT1 (SLC28A1**) identifies several SNPs to lead uptake of purines Nucleosides analog; hCNT3 (SLC28A3) allow both pyrimidines and purines transport.

CDA

It has been well demonstrated that genetic variability within the cytidine deaminase gene might be an important modulating factor of Gemcitabine and ARA-C toxicity. Three variants in the *CDA* 5' UTR promote region (-451C>T, -92A>G and del-31) lead to alterations in putative transcription factor binding sites and influencing *CDA* enzyme activity assayed in Peripheral Blood Cells (PBC) of healthy individuals[19]. In addition, the *CDA* nucleotide variant 79A>C leads to a nonsynonymous amino acid substitution in exon 1 from a Lysine to a Glutamine (Lys27>Gln), and expression assays in model organisms revealed a reduced deamination capacity for the *CDA* Gln variant[20]. Beyond the reported association for the single polymorphism with CDA mRNA expression, the reduced activity caused by the Gln variant might carry a greater relevance for CDA function and the observed effects on ara-C toxicity.

Gilbert et al have assayed the *CDA* gene and performed a haplotype analysis in an ethnically diverse sample set. They have reported 15 haplotypes with a frequency >1% among Caucasians[20] (Table 1). The most frequent haplotypes in the Caucasian population can be determined by combining genotype data from the three promoter variants -451C>T -92A>G and -31del, the coding variant Lys27>Gln and in addition the silent variant 435C>T. The most significant influence between subjects variability in ARA-C cell toxicity has been observed in all highly linked *CDA* variants, except 435C>T (which discriminates the two *CDA*2* alleles, is a silent variant, and no functional impact would be expected).

The *CDA*2A* haplotype is constitute by a combination of variant alleles -451T, -92G, -31Del and 79C (27Gln)[20].

A study performed on 100 Peripheral Blood Cells (PBC) has reported that among individuals carrying two **2A* alleles, ARA-C-induced toxicity has been approximately 53% higher when compared with carriers of no **2A* alleles and nearly 74% higher compared with carries of two wild-type **1A* alleles. The most important association

TABLE 2. SELECTION OF VALIDATED PHARMACOGENOMICS VARIANTS INFLUENCING GEMCITABINE-BASED THERAPY

	Genetic variants (Codon) dbSNP rs	Afr	Eur	Asn	Activities related SNP	Evidence in literature
GENES						
hENT1 (SLC29A1)	-706 G>C (5'UTR) rs747199	(C) 0	(C) 0.21	(C) 0.23	Major influx of nucleoside analogs inside the cells due to increased mRNA synthesys	The -706GG variant has been demonstrated to alter putative transcription factor binding sites and is associated with an increase in hENT1 mRNA expression in peripheral blood cells[16].
hCNT3 (SLC28A3)	1538A>T (Thy513Phe) rs56350726	(A) 0,13	(A) 0,07	(A) 0,1	No functional change	Association to Gemcitabine toxicity in pancreatic cancer[17].
CDA	-451C>T (5'UTR) rs532545	(T) 0,07	(T) 0,32	(T) 0,13	Low activity of promotorial 5'UTR Low mRNA level. High level of ARA-CTP resulting in bone marrow depression	PBC from healthy donors, report that among individuals carrying two **2A* alleles (see Table 1), ARA-C-induced toxicity was approximately 53% higher when compared with carriers of no **2A* alleles and nearly 74% higher compared with carries of two wild-type **1A* alleles[22]
	-92A>G (5'UTR) rs602950	(G) 0,07	(G) 0,32	(G) 0,13		
	31Del (5'UTR) rs3215400	(C) 0,33	(C) 0,43	(C) 0,44		
	79A>C (Lys27Gln) rs2072671	C) 0,08	(C) 0,33	(C) 0,13		
	435T>C (Thr145Thr) rs1048977	(T) 0,37	(T) 0,33	(T) 0,25	Low enzyme activity	
DNA REPAIR						
XRCC1	28152G>A (Arg399Gln) rs25487	(T) 0,12	(T) 0,35	(T) 0,25	Low activity in Base excision DNA repair	Mutations are correlated to prediction of better treatment outcomes in patients. In long term therapy the probability of resistance phenomena is high[23]
ERCC2 (XDP)	35931A>C (Lys751Gln) rs13181	(G) 0,21	(G) 0,38	(G) 0,09	Low activity in Nucleotide excision DNA repair	

*from Ensamble

was observed for *CDA* -31Del, where the CDA mRNA expression was 1.37-fold increased among homozygote carriers of the deletion compared with wild type carriers (no deletion -31), moreover, the presence of additional homozygous CDA 79C allele demonstrate a lethal CDA enzyme deficiency in patients "*poor metabolizer*" to cytarabine[21].

Controversial, the CDA 79C>A polymorphism has not shown a significant impact on the response rate to gemcitabine in NSCLC patients, while the wild type CDA genotype has been indeed correlated to a lower rate of incidence of severe anemia in patients taking gemcitabine[22].

These results have been confirmed by others in NSCLC where: the results have provided suggestive evidence of a favorable effect for the XPD 312Asp/Asp and XPD 751Lys/Lys genotypes with respect to overall survival rates in platinum-treated NSCLC patients. However, the CDA 27 polymorphism does not appear to affect the efficacy of gemcitabine[23].

In hematological malignancies two study involving patients with AML treated with an ARA-C-based therapy have been reported, resulting in an increase in post induction treatment-related mortality for the C allele of the 79A>C variant.

In the first study, the rate of death from causes other than AML itself has been 2.5-fold higher in the 79CC group compared with children with two wild-type alleles. No significant differences in non hematological toxicities have been observed between the genotype groups, thus indicating a potentially important role for this variant in the modulation of Nucleoside Analogs-derived hematological toxicity[24].

In the second study, Mahlknecht et al[25] have observed similar genotype frequencies for the -451C>T, -92A>G and 79A>C variants and have reported significant linkage between these allelic variants. Among 360 ARA-C-treated AML patients, homozygote carriers of the *CDA* polymorphism -451C>T have had a >50% increased risk for death in comparison with wild-type carriers. Furthermore, they have demonstrated that the wild-type alleles of the 79A>C and the -92A>G variant are associated with a lower incidence of grade III/IV liver toxicity (i.e haplotype *1A, *1B, *1C and *1D). However, in their multivariate analysis, they have identified the -451C>T variant as an independent prognostic marker for survival. In both studies, only the influence of single *CDA* variants has been studied and no haplotype analysis has been performed.

DNA REPAIR

Pharmacogenomic studies in in cancer cells have consistently shown an increased activity of nuclear protein able to remove alien nucleotides from DNA[26]. DNA repair mechanism is controlled essentially by the Base Excision Repair (BER) and Nucleotide Excision repair (NER) genes family; furthermore, genetic variants in any of these genes may modulate repair capacity and contribute to individual variation in chemotherapy response. Primary genes involved in DNA adduct restoration are the X-Ray Cross-Complementing group (**XRCC**) and Exission Repair Cross-Complementing group (**ERCC** also named XPD). In addition other genes as ATM, RAD51 and BRCA1 are described to be involved in resistance to nucleosides analog[27]. The major polymorphisms found involved in purines resistances are described in the next section.

Germaline variation in DNA repair gene encoding XRCC1 codon Arg399Gln variants has been associated to decreasing risk of toxicity[28]. In addition, two variants in ERCC2 codon Lys751Gln and Asp312Gln have been associated with better treatment outcomes in patients receiving Gemcitabine based-therapy[29]. Furthermore, Liu X[27], has demonstrated that single strand breaks induced by Pyrimidine analogs are contrasted by DNA repair complex genes that include XRCC3, BRCA2 and RAD51 in AML.

EARLY OUTLINE EVALUATION OF GENOTYPING COSTS

A multitude of platforms have been validated to assess the mutational status of the described SNPs without defining gold standard methods. Furthermore, only few studies have addressed the cost-effectiveness of pharmacogenomics testing implicated in clinical practice[30]. For example van den Akker et al[31] included thiopurine S-methyltransferase (TPMT*)* genotyping prior to 6-mercaptopurine treatment in pediatric Acute Lymphoblastic Leukemia (ALL); the mean calculated cost from 4 European countries has averaged around € 150,00. In other study, early outline of genotyping cost for "home brew" tests (based on Fluorescent allele discrimination Assay), averaged about € 20,00 per SNP[32]. A voluntary list of international laboratories (with CLIA certification in the US and CE mark in Europe) that perform genetic tests "on demand" can be found on the National Institutes of Health website under GeneTests™ [www.ncbi.nlm.nih.gov/sites/GeneTests/lab?db=GeneTests]. To the best of our knowledge, at present time there are no available tests approved by the FDA or CE for genotyping all the variants listed in Table 1. We have evaluated the cost-analysis of custom genotyping service by a random selection of 20 interrogated certified laboratories out of 91 present on the site GeneTests™ (as to July 2014); it is approximately € 120,00 per SNP, acid nucleic isolation and shipping included.

Several platforms able to address allelic discriminations (detection of DNA mutant between the two alleles) are developed for hENT1, hCNT3, CDA, and DNA-repair genes polymorphisms (Table 2), and rational selection of the best method to detect them is dependent from the specifics aims of different laboratories[33].

Fluorescent-based systems are the most diffuse platforms due to their high specificity chemistry-based detection, resulting in an easy application in routine laboratories and medium/high-throughput; drawback, they usually require multiple tube-reactions per sample due to limitation in multiplexing capabilities. Therefore, the optimal use of this platform is achieved when a relatively small panel of SNPs must be assayed in a large number of samples. PCR-based methods like Allele Specific Amplification (ASA) and Restriction Fragment Length Polymorphisms (RFLP) do not require specialized instruments (scored as very cheap) or reagents (RFLP requires specific restriction enzyme as cited in Table 1), but these technologies suffer from a significant decrease in throughput and time-labour.

TABLE 3. KNOWLEDGE-BASE OF GENOTYPE PROFILE OF GOOD/BAD PATIENTS +RESPONDING TO GEMCITABINE TREATMENT

PGx Profile	*hENT1*	*CDA*				*XDP*	*Clinical Effects*
	rs747199 **-706G>C**	**rs532545 -451C>T**	**rs602950 -92A>G**	**rs3215400 -31Del**	**rs2072671 79A>C**	rs1381 **35931A>C**	
Genotype A	GG	CC	AA	I	AA (27Lys/Lys)	AA (751Lys/Lys)	**Very low risk of toxicity** due to good Gemcitabine metabolizer showed by CDA *1A allele. **No risk of resistance** phenomena were described in hENT1 CC and XPD genomic profile.
Genotype B	GG	CC or CT		I/-31Del	AC (27Lys/Arg)	**CC** (Gln/Gln)	**Warning for resistance phenomena** due to presence of decreased activity enzyme of hENT1 and probable resistance phenomena due to Heterozy gous/homozygous XPD Gln.
Genotype C	GG or GC	CC or CT		I/-31Del	AC (27Lys/Arg)	CT or TT	**Warning for intermediated risk of toxicity and probable resistance** as consequence of variable heterozygous CDA, decreased activity enzyme of hENT1 and probable resistance phenomena due to Heterozygous/ homozygous XPD Gln.
Genotype D	GG or GC	**TT**	**GG**	**-31del**	**CC** (27Arg/Arg)	CT or TT	**Warning for increased risk of toxicity** due to Very poor metabolizer: who carrying two **2A or *2B* alleles shown a 53% increase toxicity compared with carriers of no **2A* alleles ($p = 0.03$), and a 74% elevation in comparison with carries of two wild-type **1A* alleles[22].
Genotype E	**CC**	**TT**	**GG**	**-31del**	**CC** (27Arg/Arg)	**CC** (Gln/Gln)	**Very high risk of toxicity** caused by: i) high level of intracellular Gemcitabine due to high hENT1 mRNA expression[17]; ii) low metabolization rate of Gemcitabine in CDA *2A or *2B allele; iii) High probability of resistance due to XPD Gln/Gln

Refer to Table 1 for CDA haplotype nomenclature
Letters in bold denote variant allele

High-throughput genotyping methods require specific equipment which can be rather expensive. For example, Pyrosequencing, and MALDI TOF systems require tagged primers (e.g., biotin) for pre-detection purification steps (scored as high cost). The chip-based assays have physical matrix requirements (synthesized DNA chip arrays tailored to SNPs targets) that are available only as a custom service (scored as very expensive). In each case, DNA-sequencing represents the gold standard for a definitive confirmation of results.

Furthermore, the major issues to consider for the clinical laboratories who are responsible for providing PGx services, are: i) the availability of FDA-cleared tests; ii) the current absence of public reimbursement; iii) the need for improvement of genotyping accuracy; and iv) the need to find clinical expertise to interpret laboratory data results[34,35].

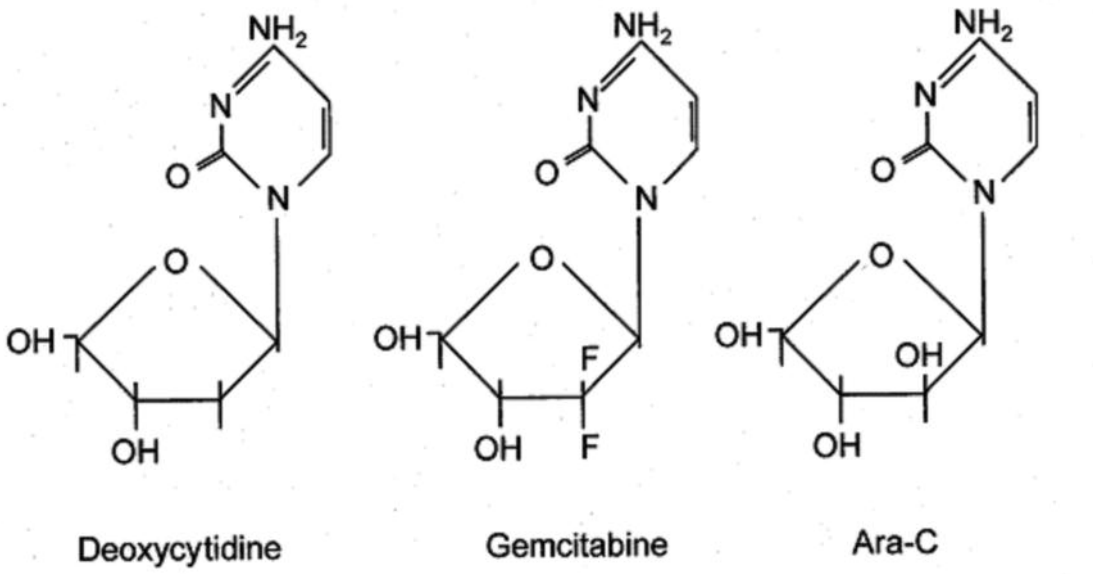

Figure 1. Pyrimidine nucleoside analogs.

CONCLUSION AND FUTURE OUTLOOK

Many clinicians recognize the usefulness of genetic tests in drug response and support the use of genetic testing to perform personalized therapy.

On the other hand, the clinical utility of the described polymorphisms involved in Gemcitabine based-therapy is in part limited by: 1. Less wide diffusion of genotyping methods in routine clinical diagnostics; 2. The evidence that PGx testing improves clinical outcomes is still an open question; and 3. The cost-effectiveness of the genotyping tests is unclear.

The useful translation from ultra-specialized laboratory into clinical practice, of the described genetic variants, will depend on their improving diagnostic prediction or fostering changes in the treatment strategies. Particularly, the molecular testing for mutation in hENT1, CDA, and DNA-repair genes, could help the oncologist to stratify patients who are most likely to minimize toxicity/resistance events. In order to assess the basic profile of drug-response (good/bad) in patients treated with Gemcitabine, a panel test of 6 SNPs is proposed (Table 3). There are some bias in our proposed panel tests: primarily these genetic variants need to be addressed experimentally in larger clinical trials including all ethnic groups; instead, the proposed panel tests is manually cured for assessing genotype among Caucasian populations. Moreover, this panel tests makes comprehensive assessment of diverse genotyping profile, arbitrarily designed from unmodified genetic germline profile called genotype A (best responders to treatment with low toxicity and low probability of resistance to drug) to genotype E (very worst responders and high probability of acute and cumulative neurotoxicity).

These issues could help the clinicians to stratify patients' profile, who showing, a favorable metabolic systems (genotype group A) because to CDA *1A haplotype and defending genetic profile to Gemcitabine resistance phenomena (i.e. hENT1, XPD, etc.).

In contrast, PGx profile described in genotype E is predisposing to very high risk of neutropenia and neuropathy showing a polymorphic variants in all genes hENT1 -706CC, CDA *2A/*2B, and XPD Lys/Lys[23]. At best of our knowledge, individuals carrying two *2A* alleles showing a 53% increase in ARA-C-induced toxicity compared with carriers of no *2A* alleles ($p = 0.03$), and a 74% elevation in comparison with carries of two wild-type *1A* alleles[36].

Furthermore, genotype groups from B to D includes intermediate ARA-C metabolizers and it could has variable effects on toxicity and sensitivity. However, SNPs in XPD are promising to elucidate the resistance phenomena oh the nucleoside analogs drugs, many Authors have found these genotyping association statistically significant[23], but many others have failed to find it[37]. We believe that the assessment of this polymorphisms should be an important predictive information of both intrinsic and acquired mechanism of toxicity/resistance in patients with intermediate enzyme activity. Results from several genotyping approach optimized for management of Gemcitabine -induced toxicity seem promising, but confirmation in larger clinical trials is still needed either for scheduling of dosage adjustment or planning other pharmacologic agents.

Over the next few years, the emergence of molecular resistance/toxicity related-therapies as results of the genomic alterations in cancer will drive diagnostics companies to develop new tests able to produce results for tailoring patient's treatment[38]. Hopefully, the future implementation of the methods for genotyping the variants influencing cytarabine-based therapy will result in personalized treatments. Therefore, it is fundamental that pharmaceutical and biotechnology companies join, in order to develop an extensive study on the standardization method to validated tests suitable for routine diagnostics in pharmacogenomics of cytarabine.

Besed on these purpose, the clinician and the lab manager may join together to evaluate advantages and limitation, in terms of costs and applicability, of the most appropriate methods to setting Gemcitabine pharmacogenomics tests.

Aknowledgements:
The authors are grateful to Dr Orlando Barletta from the Gruppo Oncologico Ricercatori Italiani (GORI) for the invaluable bibliography research, and Mrs. Paola Favetta for her expert assistant in the preparation and correction of the manuscript.

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