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How gene polymorphisms can influence clinical response and toxicity following R-CHOP therapy in patients with Diffuse Large B Cell Lymphoma

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Abstract

The treatment of diffuse large B cell lymphoma (DLBCL) is generally based on multidrug chemotherapy, for instance the therapy with rituximab together with cyclophosphamide, vincristine, doxorubicin, and prednisone (R-CHOP). A significant proportion of DLBCL patients benefit from rituximab-based chemoimmunotherapy. However, among patients with DLBCL toxic effects due to therapy treatment are still very frequent, as well as inter-individual differences in the outcomes of patients even having similar stage, histological grade and histopathological type of the tumor.

The present paper reviews the actual status of pharmacogenomics studies concerning gene polymorphisms that may affect response and tolerability to R-CHOP therapeutic regimen used to treat DLBCL. There are clear evidences that polymorphisms of genes codifying for protein are involved in cytotoxicity induced by R-CHOP regimen. Moreover, polymorphisms in genes encoding TNF-superfamily cytokines and proteins involved in controlling cellular cycle and tumor growth may be related to variability in efficacy of R-CHOP therapy in DLBCL patients.

This knowledge emphasizes the clinical meaning and importance of pharmacogenetics in oncology. The main merit of our study seems to have tried for the first time a comprehensive review of gene polymorphisms that are involved in the response to an entire therapeutic protocol, R-CHOP, in a specific disease, DLBCL, rather than examining polymorphisms referred to individual drugs among themselves not connected or used to treat different pathological conditions. Indeed, it seems clear that only the analysis of a constellation of polymorphisms can really be useful in clinical practice, while knowledge of a single polymorphism seem to give a limited contribution to our ability to use genetic analysis to the management of patients with malignant blood disorders.

Keywords: Gene polymorphisms; Pharmacogenetics; Diffuse large B cell lymphoma; R-CHOP regimen; Drug toxicity; Prognostic stratification methods.

1. Introduction

About 30% of lymphoma cases are represented by diffuse large B cell lymphoma (DLBCL), the most common type of lymphoma [1]. Although DLBCL is curable in a large part of patients, almost 30–40% of them eventually relapse or are primarily refractory within the first 2-3 years and do not achieve complete remission [2,3]. A retrospective study has shown that 30% of patients with DLBCL treated with second line therapy are truly non responders [4]; in this population the response rate (RR) to immune-chemotherapy-based third line therapy is 20% while the median overall survival (OS) is only 4 months [4]. Furthermore, patients relapsed after autologous stem cell transplant have median OS of 9 months [5]. The association of cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP) has become the standard treatment for DLBCL since the '70s and the addition, in 2002, of the chimeric anti-CD20 monoclonal antibody rituximab to CHOP (R-CHOP) appeared able to significantly ameliorate the prognosis of patients with DLBCL. In patients with relapsed/resistant DLBCL, R-CHOP increased the 5-year OS of 58% in comparison with 45% for CHOP alone [6]. Thus, the association of rituximab and CHOP (every 14 or 21 days) has since become the standard therapy for patients with DLBCL. However, over 30% of patients will fail to respond to this currently available regimen or will relapse with resistant disease. As a consequence of this incidence, even slight improvement in the outcomes of DLBCL patients has the potential for high impact on their survival [7].

The oncologist can count on a number of prognostic factors, including clinical (tumour volume, node invasion, etc) and pathological data (histoprognostic grade). All these factors are surely useful to get a therapeutic decision but are often not sufficient to predict which is the best drug or drug combination to be chosen so to obtain, for a given patient, the highest probability of therapeutic success. The anticancer drugs are often prescribed also based on patients' characteristics (age, sex, liver and kidney functions, comorbidities, etc.). The knowledge of individual genetic features, including gene polymorphisms, can help to foretell the efficacy and toxicity of a drug therapy and to offer a rational personalized drug regimen. In fact, numerous genes associated with the response to anticancer therapies have a role in pathways related to transport, metabolic activation, and detoxification of drugs. They can encode non only proteins involved in drug distribution and metabolism but also drug targets (receptors, enzymes, etc) or proteins related to oncogenesis/ oncosuppression, cell signaling, and DNA repair.

The scope of this review is to provide an up to date report on the status of clinical pharmacogenetic studies targeting to identify the associations between gene polymorphisms and the response [both as therapy efficacy (Tab. 1) and toxicity (Tab. 2)] to R-CHOP treatment in patients with DLBCL. The characteristics of the studies included in this review, such as the description of enrolled patients,

therapy, and outcomes, as well as gene polymorphisms studied, are summarized in Supplementary Table S1, while a list of nomenclature used for genes and proteins is reported in Supplementary Table S2.

2. The role of the R-CHOP therapeutic regimen in treatment of DLBCL patients

One of the main problems hindering the therapeutic success in DLBCL patients is the heterogenity of this disease, not only at the morphologic and clinical level but also at the genetic and phenotypic one [8]. DLBCL comprises specific subtypes or disease entities. As established in 1994 by the International Lymphoma Study Group [9], a single "diffuse large B cell lymphoma" category includes 3 groups of high-grade lymphomas which were originally defined as histiocytic or centroblastic lymphoma, immunoblastic B cell lymphoma, and large B cell anaplastic Ki1+ lymphoma [10-13]. However, DLBCL revealed itself as a disease more heterogeneous than the classification had initially defined. DLBCL can be subclassified based on cytologic appearance, the site of primary involvement, and according to the clinical background from which they arise (e.g., normal or compromised immunity) [14]. Importantly, these disparate features are reflected by the wide spectrum of clinical outcomes and treatment response [15,16].

As said before, DLBCL is characterized by a heterogeneous nature. In recent years, gene expression profiling (GEP) studies identified 3 distinct molecular diseases: activated B-cell (ABC), germinal center B-cell (GBC), and primary mediastinal B-cell (PMBL) DLBCL [17,18]. However, most cases are still classified as DLBCL not otherwise specified (NOS). Moreover, GEP studies identified the "cell of origin" of DLBCL cases. The GCB and ABC DLBCLs originate from B cells at different stages of differentiation; more exactly the first subtype arises from GCB cells and the latter probably from post-GCB cells blocked during plasmacytic differentiation. PMBL, which mainly affects young people, presents clinical and pathological features resembling those of Hodgkin's lymphoma (HL).

Most newly diagnosed patients with DLBCL receive rituximab in combination with a chemotherapy backbone consisting of CHOP. This backbone has been used since the early 1970s when doxorubicin was added to cyclophosphamide, vincristine, and prednisone (CVP), and CHOP became the first curative regimen in DLBCL [19]. The regimen including rituximab and CHOP (R-CHOP) became the new standard for the treatment of DLBCL in the early 2000's, based on investigations of the Groupe d'Etude des Lymphomes de l'Adulte (GELA) in DLBCL cases aged ≥ 60 years [20]. A number of further studies have been carried out by other research teams in DLBCL patients, comparing R-CHOP regimens (given every 14 or 21 days) also with other drug protocols

[for example the R-ACVBP (rituximab plus doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone)] [20-28], but on the basis of their findings R-CHOP-21 remains the standard.

As to the efficacy of R-CHOP in the different DLBCL subtypes, one has to point out that, besides relevant from a scientific viewpoint, to distinguish between ABC and GCB DLBCLs is also important for the clinical implications, since these subtypes show different OS following the current standard treatment with R-CHOP [13]. The therapy with R-CHOP is successful in the vast majority of GCB DLBCL patients but it is less effective in ABC DLBCL cases [29].

3. Gene polymorphisms

Gene polymorphisms consist of a variation in the gene DNA sequence occurring in a population with a frequency ≥ 1% [30,31] and resulting in changes in the expression, structure, and activity of the proteins encoded by these genes. As suggested by the higher frequency in the population, polymorphisms with no effect or beneficial effect are naturally occurring, and in most cases relate to genes that do not have essential functions for the life of the cell or the organism.

Single nucleotide polymorphisms or SNPs involve only one nucleotide and exemplify the commonest polymorphism, and believed to be found every 1,000 base pairs in the human genome; SNPs are generally present in areas flanking protein-coding genes (which are crucial for microRNA binding and to regulate protein expression) as well as at the level of exonic sequences, introns, and at the junction between exons and introns, at a splicing site. Furthermore, some more complex polymorphisms involve the number of mini/microsatellite repeats (variable number of tandem repeats, VNTR) or of copies of a gene (copy number variations, CNV); these polymorphisms have a main role in determining the amount of the mRNA and the protein produced. While missense polymorphisms induce the replacement of an amino acid residue by another one, and thus modify the sequence of a protein, synonymous (silent) polymorphisms may affect protein function. An explanation might be that the frequency of the various codons corresponding to the same amino acid is variable, and the related tRNA pool may not be adapted to the substitution of a codon by another one, resulting in slower protein synthesis or imperfect protein folding. Finally, insertion or deletion of one nucleotide can cause a break of the reading frame (frameshift polymorphism), which may very likely encode a truncated and totally inactive protein.

4. The drug: targets and action mechanisms

Rituximab, one of the components of R-CHOP, is a humanized IgG1 monoclonal antibody (mAb) having two domains: the fragment ab (Fab) binding the B-lymphocyte antigen CD20 (CD20) on B lymphocytes and the fragment c (Fc) binding a Fc gamma receptor (FcgR) on an effector cell [32].

Thus the binding of rituximab to both CD20-positive DLBCL cells and effector cells expressing Fc receptors, such as NK (natural killer) cells and macrophages, allows to initiate antibody dependent cellular cytotoxicity (ADCC). Although not fully elucidated, the mechanism of action of rituximab may be explained through several theories, and includes, besides ADCC, complement-dependent cytotoxicity (CDC), induction of apoptosis, and sensitization of cancer cells to the cytotoxic effects of chemotherapics [33,34] (Fig.1).

Several genetic polymorphisms have been reported for both the rituximab cellular targets (CD20 and FcgRs), suggesting that these genetic determinants might influence the response to rituximab therapy.

Recognized to be a B-cell specific marker since 1980 [35], CD20 is highly expressed on > 80% of B-cell lymphomas but absent on pro-B cells, stem cells, and normal plasma cells [36]. Although Sar et al. [37] found no association between R-CHOP response and mutation/polymorphisms of CD20 gene (namely *MS4A1*) in a small study on biopsy sample from 23 DLBCL patients, a synonymous SNP in exon 2 in the third base of the codon for I72 (rs2070770, c.216C>T) was recently reported by Ding et al. to be highly correlated with response to R-CHOP [38]. In fact, in this study on 164 patients, there was a higher OR (overall response) and CR (complete remission) in cases with CC genotype than in patients bearing the CT plus TT genotype. Furthermore, Zhang et al. confirmed that T allele of rs2070770 may be related to superior survival, in a study on 160 DLBCL patients receiving CHOP or R-CHOP [39], underlining that a lower CD20 expression might be found in patients with the TT genotype of rs2070770. It is evident that the DNA source, as well as the number of patients and their race, could explain the different results obtained by these Authors.

As to rituximab affinity to the effector cells, FcgR is a heterogeneous family of receptors, which link together humoral and cellular responses so playing a crucial role in immunity [40]. There is evidence for the existence of 3 FcgR classes (I, II, and III) and 8 subclasses, being haplotype distribution markedly different among various ethnic groups [41] (Fig. 2). Polymorphisms in FcgR genes have been reported to be able to influence efficacy of antibody-based therapies [40].

Fc-gamma RII-a (CD32a) and Fc-gamma RIII-a (CD16A) have been shown capable to activate effector cells, while Fc-gamma RII-b (CD32B) is reported to inhibit their activation. The low-affinity receptor Fc-gamma RIII-a is able to bind the Fc portion of complexed (and not monomeric) immunoglobulin (Ig) G. Fc-gamma RII-b are predominantly expressed on NK cells and macrophages, while Fc-gamma RII-a are found on neutrophils and macrophages. Previous researches demonstrated that in subjects affected by Waldenström macroglobulinemia or follicular lymphoma the efficacy of rituximab therapy can be affected by SNPs of the *FCGR3A/FCGR2A* genes [41-43].

The more frequent *FCGR3A* polymorphism is a point mutation in the extracellular domain (rs396991, c.523T>G, F175V), also known as *FCGR3A* F158V polymorphism; it results in the expression of either valine (V) or phenylalanine (F) at amino acid position 175; this polymorphism affects IgG1 binding to the receptor [44]. NK cells of VV homozygous for the rs396991 individuals have been shown to have a greater affinity to rituximab than cells of FF homozygous individuals, so triggering a more efficient ADCC [45].

Despite this, findings on the impact of the *FCGR3A* rs396991polymorphism on the outcome of DLBCL patients undergone R-CHOP treatment are conflicting and many studies have failed to demonstrate that this polymorphism may be associated to the response to rituximab-based chemotherapy in DLBCL patients [46-49]. Other research groups, however, by comparing the results obtained in Korean patients treated with R-CHOP with those obtained in patients treated only with CHOP, have established that the V allele is significantly associated to a higher complete response (CR) rate (but not to survival) to R-CHOP in comparison with the F allele [50]. Similarly, in a study on 34 DLBCL Chinese cases, Zhang et al. showed that rs396991 VV and VF individuals respond to the initial treatment with R-CHOP-21 better than FF homozygous individuals, having a longer long-term survival [51] and, in a study on 36 Turkish patients, Büyükkurt et al. evidenced that in DLBCL patients receiving R-CHOP the FF genotype group shows an OS significantly lower than the other two allele groups [52].

More interesting may appear the findings reported by Ahlgrimm et al. in 2011 [53]. In fact, this is a prospective study carried out on 514 patients, a number really larger than that enrolled in the other research (see Supplementary Table S1). They have shown that *FCGR3A* alleles had an interaction with outcome after R-CHOP, but not CHOP, being FF genotype associated with a trend toward an inferior CR rate, EFS (event-free survival), and PFS in elderly DLBCL patients [53]; however, also in this study the results do not reach the statistical significance. However, other unidentified factors, especially related to the race as well as to the applied therapeutic regimens, may justify the differences observed among the mentioned studies.

The possible correlation between *FCGR3A* polymorphism and the efficiency of rituximab-containing regimen in patient with lymphoma is supported also by the observation that this polymorphism may be associated with the development of late-onset neutropenia (LON) following rituximab treatment [54,55]. LON is a well-recognized, although rare, late complication of rituximab-based regimens, and consists of severe neutropenia (grade 3–4) that occurs after recovery of neutrophil count from last therapy and for which other causes may be excluded. Many cases of rituximab-induced neutropenia have been found among patients receiving single-agent rituximab after multiple salvage therapies [56,57]. However, LON is more frequent in patients receiving

rituximab-based immunochemotherapy [56,58,59], and occurs, after initial neutrophil recovery from therapy, in weeks 4 to 17 following last infusion. Because the rate of disease progression is significantly low in lymphoma patients developing LON, it has been hypothesized that development of LON should reflect higher potency of rituximab, thus allowing the prediction of good outcome [60].

Li et al. showed a significantly increased rate of LON in patients receiving rituximab-containing chemotherapy with VV and VF than in the FF group, being LON clinical course generally self-limiting [54]. Also Keane et al. have demonstrated a strong correlation between the rs396991 VV genotype and rituximab-induced LON development [61]. In fact, no relapse was observed in patients with either LON or rs396991 VV genotype, although there was no association with better EFS or OS after R-CHOP.

These data contribute also to formulate new hypotheses to explain the exact mechanism through which rituximab can induce neutropenia. With reference to the *FCGR3A* rs396991 polymorphism, in patients with high-affinity FcgRs rituximab could induce the ADCC on both normal and malignant B lymphocytes in a more efficient way. One hypothesis to explain why rituximab-induced neutropenia appears a self-limiting process is that neutrophils are killed by the influx of granzyme and lysozyme released by effector cells, through a bystander effect, and thus, after the target B cells are destroyed, the ADCC will not persist. Furthermore, the *FCGR3A* rs396991 polymorphism may improve the B lymphocyte depletion. The deeper B-lymphocyte depletion so occurring in subjects bearing the high-affinity VV genotype might result in leading the bone marrow in lymphopoiesis and far from granulopoiesis [62].

Also *FCGR2A* (the most widely expressed FcgR) can present a SNP (rs1801274, c.500A>G, H167R), also known as *FCGR2A*-H131R polymorphism, coding either arginine (R) or histidine (H) at amino acid position 167 in the membrane-proximal Ig-like domain and thus modulating the affinity of the receptor for IgG immune complexes. As a consequence, the most important difference between the R and H alleles is a significantly higher affinity of the H allele for human IgG2 (and, to a lower degree, for IgG1 and IgG3) [40]. With reference on the clinical relevance and impact, Fabisiewicz et al. have shown that in DLBCL patients the frequency of primary extranodal localization was related with HH genotype [48]. In most of the pharmacogenetic studies about the significance of the *FCGR2A* rs1801274 polymorphism there is a lack of effects on the response to R-CHOP protocol [46,48,50,63]. However, Alhgrimm et al. reported that the frequency of chemotherapy-induced grade 3-4 anemia was lower in the carriers of the RR genotype [53]. Since the frequency of the toxic effects might be considered as related to the efficacy of the therapy, these

data could suggest the hypothesis of a correlation between *FCGR2A* polymorphisms and clinical outcome following R-CHOP treatment in DLBCL patients.

As reported above, there is evidence that the anti-cancer efficacy of rituximab is dependent, at least in part, on complement [64,65]. C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation; it recognizes and binds the Fc portion of immune complexes, so initiating the complement cascade and activating CDC. The sole coding polymorphism (rs172378) described for the *C1QA* gene (encoding C1q) is located at position 276 (c.276A>G). While Keane et al. showed no impact of this SNP on either EFS or OS in DLBCL patients receiving R-CHOP [61], Jin et al. showed better OR, CR, and OS in patients carrying the AA genotype [66]. However, the G to A change is a synonymous polymorphism, and so does not result in an amino acid substitution; thus, further studies might clarify the mechanism through which this polymorphism influences the response of lymphoma patients to R-CHOP therapy.

One of the mechanisms involved in the antitumor effects of doxorubicin, as well as of other chemioterapic drugs, is the production of reactive oxygen species (ROS) [67] (Fig. 3). A major endogenous source of ROS is the enzyme NAD(P)H oxidase, a multimeric protein complex catalyzing the 1-electron reduction of oxygen in presence of the electron donors NADH or NAD(P)H. The occurrence of this enzyme has been demonstrated in endothelium and macrophages, and also in the myocardium [68]. The efficacy of NAD(P)H oxidase in producing superoxide and the clinical consequences of its variability may depend on gene polymorphisms [69]. Although with a number of differences, the composition of NAD(P)H oxidases share some common features among cell types. This protein contains 2 membrane-anchored subunits, a large catalytic subunit (named gp91-phox in phagocytes, and coded by the *CYBB* gene) and a smaller adjacent subunit (named p22-phox in phagocytes, and coded by the *CYBA* gene). The p22-phox subunit is the same in all cells but very likely more than 7 homologues of the gp-91phox subunit exist [70,71]. Furthermore, the activity of the NAD(P)H oxidase is affected by 4 cytosolic proteins that interact with the membrane-anchored subunits if stimulated [72], and, in neutrophils, are termed p67-phox (coded by *NCF2* gene), p47-phox (*NCF1*), p40-phox (*NCF4*), and Rac2 (a GTP-binding protein coded by *RAC2* gene).

The rs4673 (c.214T>C, Y72H) polymorphism of the *CYBA* gene alters a heme-binding site critical for the stability of the protein. A significant decrease in ROS production was demonstrated in cells from subjects with the *CYBA* rs4673 T minor allele and this can explain the reduced tumor cytotoxicity of doxorubicin-including regimens [73] (Fig. 4).

As reported by Rossi et al. the *CYBA* rs4673 TT genotype might be predictive of poorer EFS in DLBCL patients receiving R-CHOP-21, also if its prognostic significance appeared not associated

to differences in feasibility or toxicity of the immunochemotherapic treatment [74]. More interestingly, in the same paper *NCF4* rs1883112 (c.-368G>A) polymorphism was reported to protect against several kinds of clinically significant toxic effects. In fact, the *NCF4* common rs1883112 G allele is associated with a lower risk of hematologic and cardiac effects (grade 3-4 and grade 2–4 respectively). The downregulation of the NAD(P)H oxidase is regulated by the p40-phox subunit of the enzyme, encoded by the *NCF4* gene [75]. *NCF4* rs1883112 modifies the gene promoter and so affects *NCF4* expression and ROS generation [76,77] having a relevant role in R-CHOP toxic effects. In support of this, in a study by the German non-Hodgkin Lymphoma Study Group on a large group of patients affected by aggressive Non-Hodgkin lymphoma (NHL) and undergone CHOP or CHOEP therapy, the rare AA genotype of *NCF4* rs1883112 appeared related to a higher risk of chronic anthracycline induced cardiotoxicity [76]. To investigate the correlation between NADPH oxidase polymorphisms and anthracycline-induced cardiotoxicity (AIC), Reichwagen et al. carried out a study on 150 DLBCL patients that were treated with CHOP-14 or R-CHOP-14 and included 56 cases and 94 controls (sex-, dose-, and age-matched patients who developed no cardiotoxicity) [78]. The findings seem to demonstrate that *CYBA* rs4673 CT/TT and *RAC2* rs13058338 (c.108-3812A>T) TA/AA genotypes could predispose to AIC.

DNA repair systems are fundamental to correct DNA damage induced by carcinogens or anticancer drugs and represent one of the most important mechanisms related to resistance of cancer cells to cytotoxic anticancer drugs and radiation. SNPs in genes encoding DNA repair proteins can alter not only the structure but also the functional properties of these proteins [79], so resulting in DNA repair deficiency. The DNA mismatch repair protein Mlh1, also known as MutL protein homolog 1, coded by *MLH1* gene, is a major component of the mismatch repair (MMR) system which recognizes and corrects mismatched bases and small insertion/deletion loops generated during DNA replication (Fig. 5). MMR has a fundamental role in mediating doxorubicin cytotoxicity [80], due to the double genotoxic effect of DNA adducts produced by doxorubicin. In fact, these adducts can induce nucleotide mismatches and interfere with normal MMR activity, thus avoiding a complete repair of nucleotide mismatches. The accumulation of unrepaired DNA initiates apoptosis in cells characterized by MMR competence. On the contrary, MMR deficient cells become resistant to chemotherapy and continue to proliferate despite DNA damage caused by doxorubicin. The G allelic variant genotype of rs1799977 SNP (c.655A>G) is related to fewer Mlh1 proteins, thus resulting in reduction of apoptosis induced by cytotoxic drugs. As shown by Rossi et al. in DLBCL patients treated with R-CHOP-21, the death risk is increased in patients carrying the *MLH1 AG/GG* genotype in comparison with those with the AA genotype [81]. The poor prognosis of *MLH1 AG/GG* was due to the higher risk of failing R-CHOP therapy, as well as platinum-based second-

line treatment. This effect is in agreement with the role of Mlh1 in modulating the genotoxicity of doxorubicin and platinum compounds. However, opposite data are reported in a study by Melchardt et al. in a cohort of elderly DLBCL patients (> 75 years), since the *MLH1 GG* genotype was associated to a trend toward a better outcome [82]. The Authors suggest that in DLBCL the driving element for a successful therapy is an improved drug cytotoxic effect in young patients and lower toxicity in elderly patients, due to the greater susceptibility to toxicity and the increased vulnerability of older subjects in comparison with younger patients.

MGMT gene, coding for the Methylated-DNA--protein-cysteine methyltransferase, also known as O-6-methylguanine-DNA methyltransferase (MGMT), works as a DNA repair gene and thus has a role in a number of human malignancies. In fact, MGMT protein allows the removal of alkyl adducts from the O^6 position of guanine, so that gene G>A mutations are prevented. *MGMT* inactivation or loss may help cancer development and progression but may also improve sensitivity to alkylating agents such as cyclophosphamide. Several SNPs in the *MGMT* promoter/enhancer (P/E) region have been reported; the T variant of the *MGMT* P/E SNP rs16906252 (c.66C>T) has been related with the *MGMT* promoter methylation in several malignancies. However, in a cohort of 75 R-CHOP treated patients, Kristensen et al. evidenced no difference in OS and patient/disease characteristics associated to *MGMT* methylation status or to rs16906252 SNP genotypes [83]. Furthermore, a study on 53 DLBCL patients failed to demonstrate a role for *MGMT* inactivation as a marker of response to R-CHOP therapy [84]. However, an association between *MGMT* inactivation consequent to promoter hypermethylation and chemotherapy-induced grade 3-4 mucositis was demonstrated. These findings may be useful not only to direct the therapeutic choice, but also (taking into account the correlation between therapy efficacy and development of adverse effects) could support the hypothesis that genetic changes in *MGMT* methylation status can affect clinical outcome following R-CHOP treatment in DLBCL patients.

The enzyme DNA repair protein XRCC1, also known as X-ray repair cross-complementing group 1 (encoded by the *XRCC1* gene) has a crucial role in the process of base excision repair and single-strand break repair and thus is fundamental in the DNA repair pathway. Three common *XRCC1* SNPs, arranged in one haplotype block, have been described: R194W in exon 6 (rs1799782, c.580C>T), R280H in exon 9 (rs25489, c.839G>A), and Q399R in exon 11 (rs25487, c.1196A>G). Kim et al. found that the haplotype A (which contains all three wild-type alleles R-R-R) and the haplotype B (R-R-Q) are more frequent in controls and in DLBCL patients respectively; however, these *XRCC1* variant alleles appeared not associated with treatment outcomes [85].

5. The host: drug availability and metabolism

Polymorphisms in genes coding for proteins implicated in bioavailability, metabolism, and excretion of drugs can affect the outcome of patients with lymphoma. Doxorubicin is transported by the ATP-binding cassette sub-family G member 2 pump (ABCG2) [also known as breast cancer resistance protein (BCRP)]. ABCG2 pump works as an energy-dependent efflux pump transporting a broad variety of substrates across the cell membrane, so affecting intracellular concentration of anticancer drugs and both their efficacy and toxicity [86,87]. *ABCG2* gene polymorphisms rs2231137 (c.34G>A, V12M) and rs2231142 (c.421C>A, Q141K) have a high frequency in most ethnic populations [88]. Different studies described how rs2231142 in particular affects pharmacokinetic of various ABCG2 substrates [86,88,89]; this polymorphism results in lower expression and activity of ABCG2 and thus in an accumulation of ABCG2 substrates [90]. Kim et al. in a study on R-CHOP-treated DLBCL patients, reported that some severe non-hematological side effects (grade III–IV), such as fever, infection, and diarrhea occurred with a higher frequency in those carrying the non-QQ genotype [91].

The Canalicular multispecific organic anion transporter 1, also known as multidrug-resistance-protein 2 (MRP2), encoded by the *ABCC2* gene, is an ATP binding cassette (ABC) efflux transporter having among its various substrates doxorubicin and vincristine [92]. Rossi et al. reported a poorer EFS following R-CHOP in DLBCL patients carrying the *ABCC2* nonsynonymous SNP rs17222723 (c.3563T>A) AT/AA genotypes [74].

The metabolism of the anthracycline drugs and their toxicity depends, in part, by carbonyl reductases (CBRs), a phase I biotransformation enzyme which plays a major role in converting aldehyde- and ketone-containing compounds to their corresponding hydroxy metabolites [93]. The Carbonyl reductase [NADPH] 3 isoform is widely distributed in human tissues. With regard to its pharmacogenetics, the *CBR3* gene polymorphism rs1056892 (c.730G>A, V244M) affects the enzymatic efficiency, being in a region critical for the interaction with the cofactor NADP(H) [94]. Blanco et al. reported that the AA genotype is protective against anthracycline-induced cardiotoxicity in children and adolescents treated with low-moderate anthracycline doses for different types of childhood cancer, because it leads to a lower accumulation of doxorubicinol, the primary metabolite of doxorubicin, partially accounting for doxorubicin cardiac toxicity [95]. Melchardt et al. showed a similar beneficial effect of this genotype in elderly DLBCL patients, identifying a group of patients with lower levels of doxorubicinol and a significantly better OS [82]. Interestingly, this research group has defined a favorable genotype regarding toxicity. In fact, the incidence of severe leukopenia was lower in homozygous patients with *CBR3* rs1056892 AA – *MLH1* rs1799977 GG genotype, and no acute treatment related mortality (TRM) was observed in this group.

Polymorphisms of phase II enzymes of the glutathione S-transferase (GST) family have been correlated with risk of many cancers. GSTs detoxify chemotherapeutic drugs or their metabolites by increasing their water solubility through conjugation with glutathione and thus facilitating excretion, and in particular their detoxifying activity is crucial, for the efficacy and toxicity of cyclophosphamide and doxorubicin in the CHOP and R-CHOP regimens.

The *GSTT1* and *GSTM1* genes of the GST superfamily show null or deletion polymorphism [96,97], and subjects homozygous for the null allele do not show any GST enzyme activity. In the study of Cho et al. on 94 de novo DLBCL patients treated with R-CHOP, treatment response rate was not correlated to GST polymorphisms but a relevant manifestation of severe (grade III e IV) leukocytopenia, fever, and mucositis was present in patients with the *GSTT1*-null genotype with respect to patients with an undeleted *GSTT1* gene [98]. Also, patients with the *GSTM1/T1* double-null genotype showed an elevated risk of developing severe (grade III and IV) thrombocytopenia than those with other genotypes. Nevertheless, no GST genotype had any impact on OS, but there was a significantly worse EFS in males with *GSTM1/T1* double-null genotype. In addition, Yri et al. reported a superior OS in patients carrying the *GSTM1* undeleted genotype and with low International Prognostic Index (IPI), while deletions in *GSTM1* and *GSTT1* were correlated to altered susceptibility of developing DLBCL [99].

GSTA1, belonging to the alpha class of GST, is the most abundantly expressed GST in hepatocytes and kidney proximal tubules. The polymorphisms *GSTA1* rs3957357 (c.-135T>C, formerly known as -69C>T), rs3957356 (c.-118A>G, formerly known as -52G>A), and rs4715332 (c.-633G>T, formerly known as -567T>G) are located in the promoter region of *GSTA1* (in a cluster mapped to chromosome 6). These three, apparently linked, polymorphisms result in differential expression with lower levels of the enzyme in individuals with the *GSTA1*B* variant (rs4715332 G, rs3957357 T, rs3957356 A) than the common *GSTA1*A* allele (rs4715332 T, rs3957357 C, rs3957356 G). *GSTA1*B* leads to decreased detoxification of alkylating agents, with higher exposure of cancer cells [100-102]. However, Guy and coll. [101] proposed that the SNP rs186381505 (c.-182G>A, formerly known as -115C>T) also has a major effect in lowering GSTA1 levels. Thus polymorphisms of these genes could influence outcome in DLBCL patients receiving R-CHOP. In fact, Rossi et al. noticed that patients with the *GSTA1* rs3957357 CT/TT genotypes show better EFS with respect to patients with the CC genotype, the improved outcome being very likely related to higher levels of cyclophosphamide derivatives endowed with cancer cell killing activity [74]. Furthermore, Yri et al. (referring for rs3957357 to g.52803889A>G) reported that patients with high IPI score (3 – 5 factors) and with the *GSTA1* rs3957357 G allele receiving rituximab-containing therapy tend to display worse outcome with respect to those homozygous for the A allele [99].

5. The tumor and its microenvironment

5.1 The cytokine signaling pathways

Tumor Necrosis Factor (TNF) is among the first cytokines released during the inflammatory processes, and it generates a cascade involving the production of interleukin-1, interleukin-6 (IL-6), as well as other mediators, including TNF as well [103,104]. In vitro studies suggest that elevated levels of TNF-α may decrease the sensitivity of cells to apoptosis induced by some chemotherapeutic drugs [105]. The *TNF* gene is in chromosome 6, in the class III region of the human lymphocyte antigen (HLA) [106], and SNPs have been found in its promoter [107]. Of these, the rs1800629 (c.-488G>A), also known as -308G>A is the most studied [108]. *TNF* rs1800629 A allele was shown to lead to a higher transcriptional activity than the more frequent *TNF* rs1800629 G allele and to be associated with higher constitutional and inducible expression of TNF-α, and heterozygosity implicates increased TNF production [109,110].

Yri et al. have shown that *TNF* rs1800629 A allele is correlated to worse outcome only in the group of Caucasian patients not receiving rituximab [99], while Tarabar et al. have demonstrated, in a ethnically matched group of DLBCL patients, that those who carry the GG genotype were more sensitive to R-CHOP than those carrying the AG/AA genotypes, and the latter had a significantly more resistant/progressive disease at the beginning of the therapy [111]. One has to remember that rituximab may be efficiently employed in adults with rheumatoid arthritis who had a poor response to one or more anti-TNF-α therapies. The results by Al-Zoobi et al. point out the role of the association between CD20 and CD40 (member of the TNF receptor family physically associated with CD20 on cell surface) in triggering B-cell depletion [112].

In line with these findings, also some polymorphisms regarding lymphotoxin-α (LT-α), a member of TNF family, have been reported as related to the outcome in DBLCL patients treated with R-CHOP. LT-α is located in the major histocompatibility complex (MHC) III region of chromosome 6. The LT-α variant alleles are correlated with higher circulating LT-α levels and with a more severe outcome of lymphoid malignancies [113-115].

The rs909253 (c.-9-198A>G) polymorphism in the coding region of the LT-α gene leads to the wild-type A allele and to the G variant allele. In an old study carried out on 1998, Warzocha et al. studied the impact of the *TNF* (rs1800629) and LT-α (rs909253) polymorphisms on patients with DLBCL under CHOP or CHOP-like regimen, and showed that there is a higher frequency of the alleles *TNF* A and LT-α A in patients with higher TNF plasma levels, and that the presence of at least two *TNF* or LT-α "high-producer alleles" (AA) constitutes a risk factor for first-line treatment failure [116]. Also the rs1041981 SNP (c.179C>A, T60N) is correlated with the transcriptional

regulation of *LTA* that may activate the lymphocytes and induce apoptosis [117]. Chae et al. have found a correlation between this variant and time to progression (TTP) in 90 DBLCL patients receiving R-CHOP, with an inferior survival of patients with the AA genotype [118]. Conversely, in a recently published study Zhang et al. suggest that also the *LTA* SNP rs1800683 (c.-162G>A) is significantly correlated to the risk of progression or relapse in DLBCL cases but without correlation between this polymorphism and response to chemotherapy [119].

On the light of the data reported before, and since constitutive NF-kB activation can support continued lymphocyte proliferation, survival, and apoptosis [116,120], and TNF and LT-α activate NF-kB, which, in turn, may further perpetuate their autocrine production, it is not surprising that polymorphisms of the receptor-interacting serine/threonine-protein kinase 1 (Ripk1) may influence the outcome following R-CHOP therapy. In fact, Ripk1, whose activation follows binding of the TNF superfamily to its receptors, is crucial in the signal transduction of TNF-induced NF-kB activation in B cells [121]. *RIPK1* polymorphisms may be associated to an alteration of NF-kB activation in apoptosis or in immune response of lymphoma cells [118]. Chae et al. have concluded that GG genotype of *RIPK1* (rs2272990) is a negative predictive marker for R-CHOP treatment of DLBCL, connected significantly with a worse TTP [118].

Although some researchers believe that polymorphisms in the gene codifying for NF-kB can increase production and release of LT-α, causing a deregulation of NF-kB signaling, no evidence support this hypothesis. A 4 base pair ATTG insertion/deletion variant (-94ins/delATTG, rs28362491) can be found in the promoter of NF-kB1, leading to 3 different genotypes: wild-type homozygous insertion (ins/ins), variant homozygous deletion (del/del), and heterozygous (ins/del) [122]. The 4 bp deletion causes loss of binding to nuclear proteins and decreased promoter activity [122]. Giachelia et al. reported that Caucasian DLBCL patients carrying this deletion allele had higher levels of IL-6 and interleukin-10 (IL-10) [123]. Nevertheless they failed to find a direct effect of the NF-kB1 polymorphism on prognosis in patients treated with rituximab-combined therapy.

SNPs in genes codifying for IL-10 and IL-6 or for other proteins involved in cell pathways regulated by these cytokines may be correlated with risk and prognosis of Hodgkin and non-Hodgkin lymphomas [124-131]. IL-6 expression increases the expression of adhesion molecules on endothelial cells and of growth factors, thus IL-6 seems to stimulate microenvironment activity promoting cancer progression [132,133]. B and T lymphocytes and monocytes produce the pleiotropic cytokine IL-10 [134-137], a cytokine acting as a growth factor for normal activated human B lymphocytes and B lymphoma cell lines [138,139], controlling the balance between cellular and humoral immune responses, and having a strong immunosuppressive activity. Higher

serum IL-10 levels have also been found to be correlated to poor prognosis of the patients with NHL and HL [140-142], and increased production of this cytokine in the tumor microenvironment may be protective [143]. IL-10 regulates expression, in hematopoietic cells and in lymphoma cells, of bcl-2, a member of the Bcl-2 family of proteins regulating apoptosis [144]. Bcl-2 has a key role in tumor cell ability to survive cytotoxic stimuli and confers resistance to various apoptotic stimuli [145-147]. Jung et al. stated that bcl-2 overexpression may be correlated to the progression of T-cell NHL through the interaction with p53-dependent pathway [148]. IL-10 may be involved in the apoptosis-related resistance mechanism of T-cell NHL through a bcl-2 mediated pathway [149,150]. Increased expression of bcl-2 and/or IL-10 in some DLBCL patients can in part account for failure of CHOP therapy, and some studies suggest that rituximab may modulate cellular and molecular signal transduction pathways regulating bcl-2-expression, in particular in bcl-2-positive DLBCL patients [33].

The promoter region of the *IL10* gene, located on chromosome 1 (1q31–1q32), contains three common SNPs: rs1800896 (c.-1117A>G), rs1800871(c.-854T>C), and rs1800872 (c.-627A>C). Only rs1800896 seems to affect cytokine levels, with the A and G alleles associated with lower and higher levels, respectively [151]. Specific haplotypes of these three SNPs are also associated with IL-10 production levels; e.g., the *IL10* rs1800896 G, rs1800871 C, rs1800872 C (GCC) haplotype of the promoter region is associated with high expression, whereas the ATA haplotype with low IL-10 levels in children [152]. Other polymorphisms (rs1800890, rs6703630, and rs6693899) are located in the distal promoter region [153].

However, in Caucasian DLBCL patients, *IL10* promoter genotypes or haplotypes did not affect IL-10 plasma levels [123]. With reference to clinical relevance, while rs1800871 and rs1800872 SNPs had no impact on disease characteristics [154], rs1800896 SNP has been correlated to altered susceptibility of developing DLBCL in Caucasians [99]. Furthermore, a positive association has been found between *IL10* rs1800890 (c.-2692T>A) TA/AA genotypes and the presence of B symptoms in an ethnically matched groups of patients [111], but no difference in OS or EFS in relation to *IL10* rs1800890 T/A and *IL10* rs1800896 -polymorphisms was shown in this group [111].

Finally, Park and coworkers reported that rs1800871 and rs1800872 significantly influenced the overall response rate (ORR) in Korean patients receiving CHOP but not in those receiving R-CHOP [154]. More interestingly, in this same study [154], DLBCL patients were divided in 2 groups depending on SNP detection to study the interaction between *BCL2* and *IL10* SNPs: 1) a high *BCL2* and *IL10* inducible group with both rs2279115 (c.-938C>A) AA *BCL2* genotype and 2 copies of CC *IL10* haplotype for loci rs1800871 and rs1800872 (*BCL2 High/IL10 High*); 2) the low *BCL2* or

low *IL10* inducible group with rs2279115 AC/CC *BCL2* genotype or 0 to 1 copy of CC *IL10* haplotype for loci rs1800871 and rs1800872 (*BCL2 Low* and/or *IL10 Low*). Patients in the first group (*BCL2 High/IL10 High*), with a prognosis predicted to be worse than in patients included in the second group (*BCL2 Low* and/or *IL10 Low*), had significantly worse FFS and higher risk of progression in the CHOP-treated subgroup but not in patients treated with R-CHOP. In this sense, the benefits derived from adding rituximab to CHOP was enhanced in *BCL2 High/IL10 High* subgroup (in particular regarding FFS or probability of progression). Also Alas et al. supposed that rituximab-induced inhibition of IL-10-mediated loops may down-regulate bcl-2 expression and make lymphoma cells more sensitive to cytotoxic therapy [155-157]. Consequently, interaction between bcl-2 expression and IL-10 growth factors may participate to the resistance mechanisms of DLBCL to chemotherapy, and preventing this with rituximab can be a possible mechanism accounting for its therapeutic action.

Finally, a lacking of evidence is reported concerning outcomes of patients treated with R-CHOP and polymorphisms of *IL6* gene. The rs1800795 (c.-237C>G) polymorphism within the promoter region of *IL6* has been shown to influence IL-6 production in vivo, with decreased and increased expression associated with the G and C alleles, respectively [158]. Giachelia et al. confirmed that in Caucasian DLBCL rituximab-treated patients, IL-6 but not IL-10 levels are markers for outcome, measured as PFS and OS, although they found no genotype–phenotype association in DLBCL patients between IL-6 levels and the *IL6* rs1800795 SNP [123].

Human leukocyte antigen G (HLA-G) is a non-classical MHC class I molecule and due to its immunotolerant properties has a relevant role in the recognition of cancer cells by the immune system [159,160]. In the evasion phase, cancer cells lack molecules relevant for the immune identification and tend to express only HLA-G on their surface; this expression is controlled by environmental factors, such as hormones and cytokines (including IL-10). Furthermore, patients with lymphoproliferative neoplasms have elevated plasma levels of soluble HLA-G (sHLA-G). This points out that augmented expression of HLA-G facilitates evasion from immune system recognition leading to cancer cell survival [161].

Two polymorphisms in the *HLA-G* gene, rs66554220 (a 14-bp del/ins in exon 8 in the 3' UTR region) and rs1233334 (-725C>G>T in the 5' upstream regulatory region), have been shown to be functionally important and to significantly affect *HLA-G* expression [162,163].

In a recent study, in DLBCL patients of Caucasian origin treated with R-CHOP or R-COP the frequencies of the *HLA-G* rs1233334 GG or the GC genotype were lower, and the *HLA-G ins/ins* genotype was more frequent in patients with respect to controls [164]. Patients with the *HLA-G* rs1233334 CC genotype had a higher probability of OS than those with other genotype

combinations. The homozygous *HLA-G* rs66554220 del/del presented a lower probability of OS than those with the rs66554220 del/ins or the ins/ins genotype. So there is evidence of a relevant role of *HLA-G* polymorphisms for the incidence and clinical course of DLBCL; however, with reference to response to pharmacological treatment, neither *HLA-G* rs1233334 nor rs66554220 affected the probability of achieving remission after first-line treatment or response to salvage treatment.

6.2 The p-53 pathway

Activation of the tumor suppressor p53 (encoded by *TP53*) resulting from cell stress induces cell-cycle arrest and/or apoptosis and DNA repair [165] (Fig. 6). Oncogenic mutations of the p53 affects protein function so increasing genetic instability of cancer cells and, then, their tumorigenic and invasive characteristics [166]. Given that the anticancer properties of chemotherapeutics and the mechanisms involved in the effects of mAbs are achieved through activation of apoptosis, impaired p53 function may induce resistance to the current immunochemotherapy strategies in DLBCL [167]. Mutations in the *TP53* gene have been shown to confer a negative effect on survival in DLBCL [168].

TP53 intron 6 polymorphism rs1625895 (c.672+62A>G, named IVS6+62A>G) is related to altered white blood cells apoptosis in cancer patients [169]. Although it is unclear if rs1625895 affects splicing or transcription factor binding or if it is a microRNA target, it has been described as able to modulate p53 protein levels [170].

Recently, Voropaeva et al. have reported the probability of R-CHOP therapy failure in Russian DLBCL subjects with *TP53* rs1625895 GG genotype [171]. Li et al., instead, demonstrated that sequence variation in the *TP53* 3'-UTR has a prognostic importance in DLBCL and the role of this depending on the status of the coding sequence (CDS) of *TP53* [172]. In fact, by examining tumor specimens from 244 DLBCL patients treated with R-CHOP, no substantial differences in OS or PFS were associated with variation in the 3'-UTR; but better 5-year survival rate was noted in patients carrying a wild-type (WT) *TP53* CDS and 1 or more single nucleotide variants in the 3'-UTR than in patients carrying a WT CDS and the reference 3'-UTR (without statistically significance difference in OS) and there was a poorer OS in patients with 3'-UTR variation and a mutant *TP53* CDS.

The E3 ubiquitin-protein ligase Mdm2, also known as murine double minute 2 (Mdm2) protein, is a regulator of p53 activity, modulating p53 stability via a negative feedback. Higher p53 levels upregulate Mdm2, which is able to bind p53 and acts as an E3 ubiquitin ligase for proteosomal degradation of p53 [173,174]. Mdm2 overexpression has been shown to facilitate B-cell

lymphomagenesis in vivo and to inactivate the tumor suppressor function of WT-p53 in vitro [175,176].

Studies on p53 stress response pathway revealed a functional polymorphism in the *MDM2* intronic promoter (rs2279744, c.14+309T>G, also known as SNP309, 30% of allele frequency) [177]. It has been reported that the variant allele increased affinity of transcriptional activator Sp1, resulting in Mdm2 overexpression and then reduction of the p53 pathway. This kind of polymorphism has been correlated to enhanced cancer formation both in hereditary and sporadic tumors [178]. In fact, Hedström et al. reported that DLBCL patients with TT genotype were diagnosed at a younger age when compared to subjects with at least one G allele; they had considerably longer OS, lymphoma-specific survival and disease-free survival. Of note, the TT genotype was associated with a considerably longer OS in subjects not treated with rituximab, while in patients treated with rituximab no significant OS difference was observed [179]. These data are in line with those reported by Xu-Monette et al. [168] in a study on DLCBL patients treated with R-CHOP. These Authors showed that there is no significant difference in the mean Mdm2 expression levels in patients with different genotypes, but p53 or Mdm2 overexpression predicts a significantly poor survival in patients with mutant-p53 but not in patients with WT-p53 [168].

DAP kinase 1 (Death-associated protein kinase 1) is a calcium/calmodulin–regulated protein kinase able to induce death cell signaling activated by IFN-γ, TNF-α, and TGF-β [180-182] through p53-dependent pathways [183]. DAP kinase 1 acts as a tumor suppressor and its levels are commonly lost in cancer and cancer cell lines. DAP kinase 1 has also been shown to be regulated at the transcriptional and translational levels by methylation of its promoter CpG island and by microRNAs, respectively [184]. In several haematological malignancies, including DLBCL, DAP kinase 1 coding gene *DAPK1* undergoes DNA methylation-mediated silencing during tumorigenesis.

Recently, Kristensen et al. have studied the effects on OS and disease-specific survival (DSS) for *DAPK1* methylation and *DAPK1* allelic methylation patterns, by genotyping the rs13300553 SNP (c.-108-612A>G). *DAPK1* promoter methylation was correlated with shorter OS and DSS in DLCBL patients [185]. When patients heterozygous for the rs13300553 are considered, SNP monoallelic methylation of the A allele was associated with shorter OS and DSS.

6.3 The vascular endothelial growth factor (VEGF) pathway

Angiogenesis is an important process involved in cancer growth and metastasis in both solid and hematologic cancers. The VEGF pathway is considered the main modulator of this process [186]. VEGF is released by many cancer cells and tumor-associated stromal cells and activates two related

tyrosine kinase receptors, VEGFR-1 and VEGFR-2. VEGFR-2 is the predominant modulator of the angiogenic effects of VEGF [186].

The gene encoding VEGFR-2, *KDR*, is highly polymorphic. The *KDR* rs1870377 SNP (c.1416A>T, G472H) produces an amino acid modification, from glutamine to histidine, in the immunoglobulin (Ig)-like extracellular domain 5. This alteration may reduce VEGF affinity for VEGFR-2. Moreover, the *KDR* rs1870377 polymorphism may be correlated to an altered downstream signaling after VEGF activation. In the study of Kim et al., although not associated with factors related to patient or cancer, this SNP was significantly correlated with both OS and PFS in Korean DLBCL subjects receiving R-CHOP [186]. In fact, patients with the *KDR* AA + TA genotypes showed significantly better OS and PFS when compared to TT genotype subjects.

6. Conclusions

The more common treatment for DLBLC is based on multiagent chemotherapy, including rituximab plus cyclophosphamide, vincristine, doxorubicin, and prednisone. Even if many patients benefit from rituximab-based chemoimmunotherapy and these active chemotherapeutic drugs have been available for many years, inter-individual efficacy and toxicities are still prevalent among patients with DLBCL, usually showing different treatment outcomes even when they have similar stage, histological grade, and histopathological type of tumor, and thus there is a medical need for a more effective therapy for relapsed/refractory DLBCL and for patients at high risk.

Pharmacodynamic proteins interfere directly or indirectly with the effect of drugs on response mechanisms. The present review summarizes and discusses the present status of pharmacogenomics studies concerning gene polymorphisms that may modify response and tolerability to R-CHOP therapeutic regimen used to treat DLBCL (see Supplementary Table S1).

We reported that polymorphisms within candidate genes involved in pharmacodynamic effects of drugs included in R-CHOP protocol can, at least partially, justify the outcomes variability with this therapeutic regimen, and thus might be predictive markers for treatment outcome with R-CHOP in DLBCL patients. In fact there are evidences that polymorphisms of genes codifying for protein involved in rituximab induced ADCC and CDC (CD20, Fc-gamma RIII-a, Fc-gamma RII-a, and C1q) can affect therapy activity, as well as also genes codifying for proteins affecting ROS production and DNA repair (p40-phox, p22-phox, and Mlh1) and so having a role in doxorubicin cytotoxicity. Interindividual differences in the pharmacokinetic processes are known to have serious implications for pharmacotherapeutic treatment and may be related to genetic polymorphisms within genes that encode proteins associated to drug bioavailability. In fact, several candidate polymorphisms have been found within genes codifying for proteins associated to drug

bioavailability, including cell efflux transporters (such as ABCG2 and MRP2, which affect cell uptake of doxorubicin and vincristine), phase I (CBR3, which is involved in biotransformation of doxorubicin) and phase II drug metabolizing enzymes (GSTM, GSTT, and GSTA1 isoenzymes, involved in detoxification of doxorubicin and cyclophosphamide). These polymorphisms can affect the safety, but also the efficacy, of R-CHOP therapy. Finally, tumor-related proteins may be also involved and affect the response to antineoplastic drugs. It was reported that polymorphism in genes encoding TNF-superfamily cytokines (TNF-α and LT-a), proteins involved in controlling cellular cycle (p53 and Mdm2) as well as in the NF-kB pathway (RIPK1) and in angiogenesis (VEGFR-2) may be related to variability in efficacy of R-CHOP therapy in DLBCL patients.

These observations underline the significance and clinical relevance of pharmacogenetics in oncology, and suggest the need for additional pharmacogenetic studies in this field, with the ultimate goal to reach a personalized therapy with better efficacy and/or lower risk of adverse effects. However, we have to observe that in some cases conflicting data are reported in literature and in some others the results are not robust enough to be conclusive. Furthermore, the causal relationship between the genetic variation and the phenotype, and the role or the effect of a polymorphism on a clinical feature can be clearly established only in some cases. This can depend on many issues. First, some factors that may influence chemotherapy response and can interfere with the data obtained from this type of analysis are well evident, in particular the protocol of drug administration (for example, R-CHOP-14 or R-CHOP-21), the administration of other therapies (including radiotherapy, stem cell transplantation and ancillary therapies), the ethnic origin of the patients (Asiatic, Caucasian, etc), the age of the patients (being elderly patients more vulnerable and prone to toxicity than younger patients), the study (design, number of patients, presence of a control group, endpoints used, etc, as well as the technology employed for DNA analyses). Furthermore, it is known that some variations are transmitted as haplotype blocks, as in the case of *IL10*.

In our case, that is the investigations on the relationship between gene polimorphisms and efficacy/toxicity of R-CHOP therapy in DLBCL, there are two critical issues that appear evident (see Supplementary Table S1). Many of the trials reported by the international scientific literature about this subject have been performed on only a few tens of patients, and, what is more, in the case of some genes and their respective proteins there are only one or two studies. Moreover, the results of different studies might be of interest to a specific ethnic group (Koreans, Chinese, etc) rather than for the overall population, and might be influenced also by other unidentified characters present in a race.

Secondly, DLBCL is a heterogeneous disease, comprising 3 distinct molecular subtypes (GBC, ABC, and PMBL). The GCB subtype is characterized by a range of genetic aberrations, including

the t(14;18) translocation, amplification of the miR-17-92 cluster, and mutations. The ABC subtype is characterized by a different genetic background that involves the t(3;14) translocation, trisomy 3, deletion of the INK4A-ARF locus, and *BCL2* amplification [187]. PMBL lymphoma is characterized by JAK2 amplification in almost half of the cases and the SOCS1 deletion, a JAK signaling suppressor, contributing to the activation of the JAK2-STAT6 signaling pathway. This heterogeneity reflects in interindividual differences in therapy outcomes but can also influence the results of pharmacogenetic studies. Further investigations on specific DLBCL subtypes, surely useful to clarify this point, are difficult to be realized, due to the number of patients to be enrolled in such trials to reach significant results.

Furthermore, one has also to mention the importance that the used DNA source may have in these studies. In fact, DNA analysis for pharmacogenetic studies is typically executed with germline DNA, using blood samples, and then appropriate for both PK and PD association analyses. On the other hand, in oncology, examination of cancer tissue (somatic DNA) is particularly interesting in PD effects evaluation, such as cancer response. In fact, genetic features of the tumor cells can affect the therapy effectiveness because of their higher genetic instability often developing additional somatic genetic modifications with genotype differences not present in non-neoplastic germ cells.

Finally, a single genetic feature is not sufficiently able to describe the interindividual differences in drug response. This is probably due to the complexity of the pharmacological pathway of a drug, with the involvement of many pharmacokinetic and pharmacodynamic proteins. Differences in response to anticancer drugs are mainly polygenetic traits. For instance, a drug may be extensively metabolized by various enzymes, or, on the pharmacodynamic level, the mutual activity of many proteins together, like signal transduction pathways and receptors, regulates the response to a drug. Moreover, in the case of chemotherapeutics, specific tumor-related proteins are also involved, and tumor progression is a dynamic process while SNPs are static indicators. Due to this complexity, the result of a single genetic modification is not sufficiently predictive of response to a drug.

Knowing in advance the effectiveness of a given drug or of a specific combination of drugs in a given patient could constitute the discriminating factor to ensure therapeutic success, as well as an analysis of polymorphisms could be useful for the prediction of the entity of the side effects of therapy.

The main merit of our study seems to have tried for the first time a comprehensive review of polymorphisms involved in the clinical response to an entire therapeutic protocol, R-CHOP, in a specific disease, DLBCL, rather than examining polymorphisms referred to individual drugs among themselves not connected or used to treat different pathological conditions. Indeed, it seems clear that only the analysis of a constellation of polymorphisms can be really useful in clinical practice,

while knowledge of a single polymorphism can give a limited contribution to our ability to use genetic analysis in the management of patients with malignant blood disorders. Further studies will perhaps determine whether an analysis of the most important polymorphisms mentioned in this review can usefully complement the current prognostic stratification methods used in DLBCL or even whether it can be used to direct appropriate therapeutic choice.

Practice points

- The treatment for diffuse large B cell lymphoma (DLBCL) is generally based on multiagent chemotherapy, including rituximab plus cyclophosphamide, vincristine, doxorubicin, and prednisone (R-CHOP).
- By the reviewing of the international literature the existence of a correlation between gene polymorphisms and the response to this therapeutic protocol in DLBCL is evident.
- Polymorphisms within candidate genes involved in pharmacodynamic effects and pharmacokinetic processes of drugs included in R-CHOP protocol, as well as polymorphisms in genes encoding tumor-related proteins, may affect efficacy and/or toxicity of R-CHOP regimen in DLBCL.
- Since in some cases findings are conflicting or not robust enough to be conclusive, further additional pharmacogenetic studies in this field are absolutely required.

Research agenda

- Further pharmacogenetics investigations on specific DLBCL subtypes.
- Relationship between R-CHOP response and polymorphisms in genes encoding tumor-related proteins.
- Development of a prognostic score by gene polymorphism analysis.
- Consider pharmacogenetic analysis in the therapeutical decisional process address.

Conflict of interest

None.

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Figure captions

Fig. 1. The mechanism of action of rituximab proposed, including antibody dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), induction of apoptosis and chemosensitization of tumor cells to the cytotoxic effects of chemotherapy.

Fig. 2. The family of Human Fc receptors for IgG (FcRs). An activating FcR (Fc-gamma RII-a and Fc-gamma RII-c) consists of a ligand-binding-chain and a signal-transducing-chain dimer, which carries immunoreceptor tyrosine based activating motifs (ITAMs). Fc-gamma RII-b contains an immunoreceptor tyrosine based inhibitory motif (ITIM) in its cytoplasmic domain.

Fig. 3. The metabolism of the anthracycline drugs and their toxicity mechanisms. Abbreviations: CBR1, carbonyl reductase 1; CBR3, carbonyl reductase [NADPH] 3; CYBA, cytochrome b-245 light chain; CYP2J2, cytochrome P450 2J2; MDR1, multidrug resistance protein 1; MRP1, multidrug resistance-associated protein 1; MRP2, multidrug resistance-associated protein 2; NCF-4, neutrophil cytosolic factor 4; NQO1, NAD(P)H dehydrogenase, quinone 1; PPARα/β, peroxisome proliferator-activated receptor γ co-activator 1-α/β; Rac2, ras-related C3 botulinum toxin substrate 2 (rho family, small GTP-binding protein Rac2); ROS, reactive oxygen species; TOP2B, topoisomerase 2β; p53, cellular tumor antigen p53.

Fig. 4. NADPH oxidase complex is a cluster of proteins that donate an electron from NADPH to molecular oxygen (O_2) to produce superoxide (O_2-). Two transmembrane subunits, gp91-phox and p22-phox represent the catalytic core of the complex and catalyze the transfer of electrons.

Fig. 5. The mismatch-repair (MMR) system greatly contributes in maintaining genetic stability of eukaryotes and prokaryotes. Proliferating cell nuclear antigen (PCNA), a DNA clamp that prevents DNA polymerase dissociation from the DNA template during replication, interacts with MutS homologues (MSHs) complex, which indicates that MMR might occur during DNA replication. Single-stranded DNA breaks occur during MMR, the lesion is digested by exonucleases, such as EXO1, and then filled-in by DNA polymerases.

Fig. 6. The p53 transcription factor activates target genes promoting cell-cycle arrest or apoptosis. Normally, Mdm2 maintains low levels of p53 by targeting the protein for proteasome degradation, and by directly blocking p53 transcriptional activity. Furthermore, *MDM2* is inhibited by ARF, one

of two Alternative open Reading Frame tumor-suppressor products produced from the p16INK4A-ARF locus. DAP-kinase 1 (DAPK1), a serine/threonine kinase, induces p53 activity directly. An indirect mechanism of DAPK1 dependent p53-activation involves activation of the ARF tumour suppressor.

Table 1. Polymorphisms reported to influence efficacy of R-CHOP therapy in DLBCL patients.

Protein (gene)	Function	Polymorphism	References
CD20 (*MS4A1*)	Rituximab receptor	c.216C>T, rs2070770	[37-39]
Fc-gamma RIII-a (*FCGR3A*)	Rituximab receptor	c.523T>G, F175V, rs396991	[46-54,61]
C1q (*C1QA*)	Involved in rituximab CDC	c.276A>G, rs172378	[61,66]
p22-phox (*CYBA*)	Subunit NAD(P)H oxidase, involved in ROS production and doxorubin cytotoxicity	c.214T>C, rs4673	[74]
Mlh1 (*MLH1*)	Component of DNA MMR system, involved in mediating doxorubicin cytotoxicity	c.655A>G, rs1799977	[82]
MRP2 (*ABCC2*)	Cell efflux system for doxorubicin and vincristine	c.3563T>A, rs17222723	[74]
CBR3 (*CBR3*)	Phase I biotransformation enzyme, involved in doxorubicin biotransformation	c.730G>A, rs1056892	[82]
GSTA1 (*GSTA1*)	Phase II biotransformation enzyme, involved in detoxification of cyclophosphamide	c.-135T>C, rs3957357	[74,99]
TNF-α (*TNF*)	Cytokine produced in inflammatory processes	c.-488G>A, rs1800629	[99,111]
LT-α (*LTA*)	Member of TNF family	c.-9-198A>G, rs909253	[116]
		c.179C>A, rs1041981	[118]
Ripk1 (*RIPK1*)	Mediating signal transduction of TNF-induced Nf-kB activation	+83G>A, rs2272990	[118]
p53 (*TP53*)	Tumor suppressor	c.672+62A>G (named IVS6+62A>G), rs1625895	[171]
		TP53 3'-UTR (predominantly G/A and C/T substitutions)	[172]
		TP53 coding sequence (CDS) mutations	
Mdm2 (*MDM2*)	Modulating p53 stability	c.309T>G, rs2279744	[168,179]
DAP kinase 1 (*DAPK1*)	Tumor suppressor	rs13300553	[185]
VEGFR-2 (*KDR*)	Involved in angiogenesis	c.1416T>A, rs1870377	[186]

Table 2. Polymorphisms reported to influence toxicity of R-CHOP therapy in DLBCL patients.

Protein (gene)	Function	Polymorphism	Effect	References
Fc-gamma RII-a (*FCGR2A*)	Rituximab receptor	c.500A>G, H167R, rs1801274	Less frequent grade 3-4 chemotherapy-induced anemia in RR genotype	[53]
p40-phox (*NCF4*)	Cytosolic protein affecting NAD(P)H oxidase activity involved in ROS production and doxorubin cytotoxicity	c.-368G>A, rs1883112	G allele associated to reduced risk of grade 3–4 hematologic and grade 2–4 cardiac toxicity	[74]
p22-phox (*CYBA*)	Subunit of NAD(P)H oxidase, involved in anthracycline-induced cardiotoxicity	c.242C>T or H72Y, rs4673	*CYBA* rs4673 CT/TT genotype could predispose to anthracycline-induced cardiotoxicity	[78]
Rac2 (*RAC2*)	Cytosolic protein affecting NAD(P)H oxidase activity involved in anthracycline-induced cardiotoxicity	c.108-3812A>T, rs13058338	*RAC2* rs13058338 TA/AA genotype could predispose to anthracycline-induced cardiotoxicity	[78]
ABCG2 (*ABCG2*)	Cell efflux system for doxorubicin	c.421C>A, Q141K, rs2231142	More frequent fever, mucositis, infection and diarrhea in non-QQ genotype	[91]
GSTM1 (*GSTM1*)	Phase II biotransformation enzyme involved in detoxification of doxorubicin and cyclophosphamide	Deletion	Higher risk of severe thrombocytopenia in *GSTM1/T1* double-null genotype	[98,99]
GSTT1 (*GSTT1*)	Phase II biotransformation enzyme involved in detoxification of doxorubicin and cyclophosphamide	Deletion	Higher risk severe leukocytopenia, fever and mucositis in null-genotype	[98]
MGMT (*MGMT*)	Removes alkyl adducts from the O^6 position of guanine	Promoter hypermethylation	Higher risk of severe mucositis	[84]

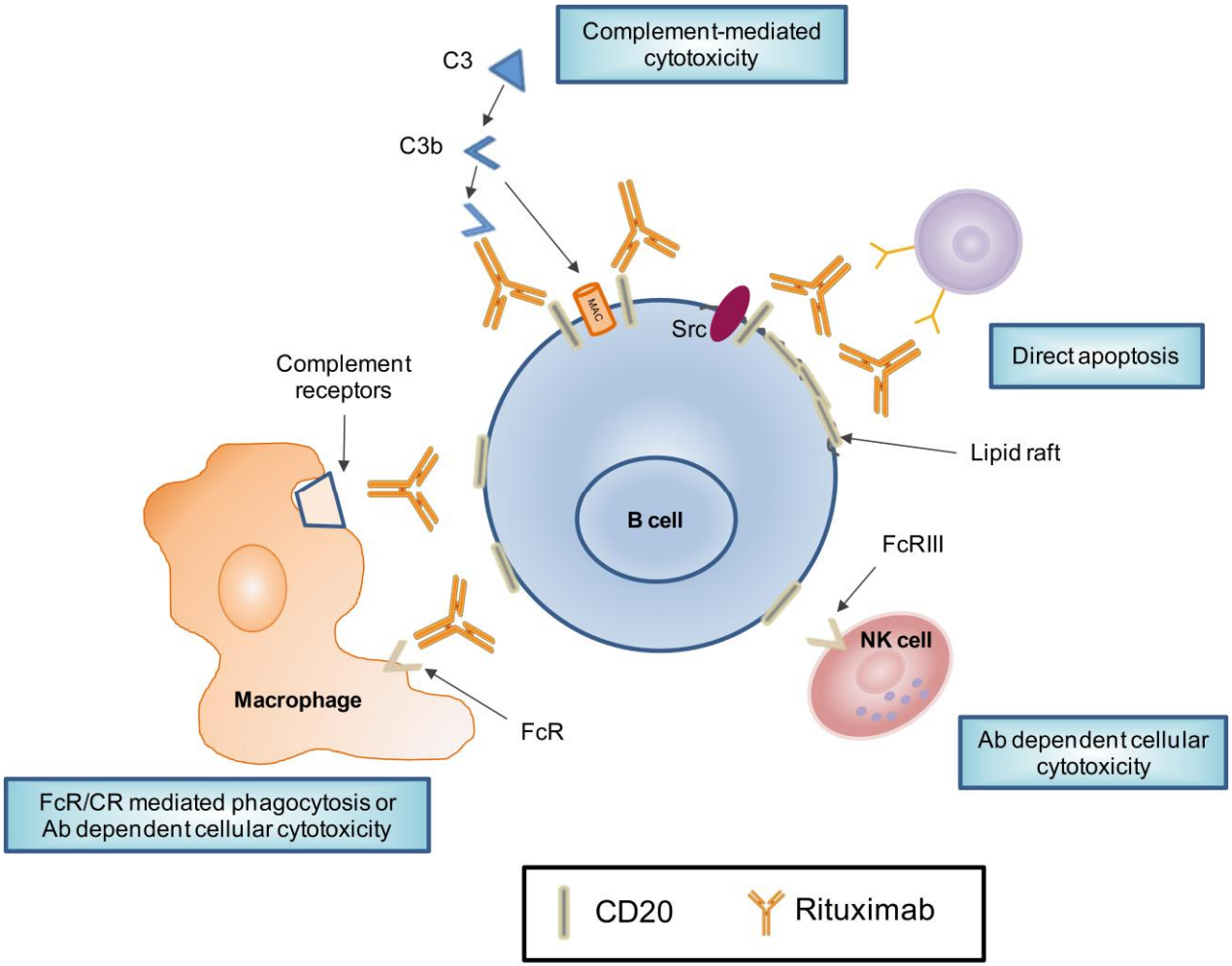

Figure 1

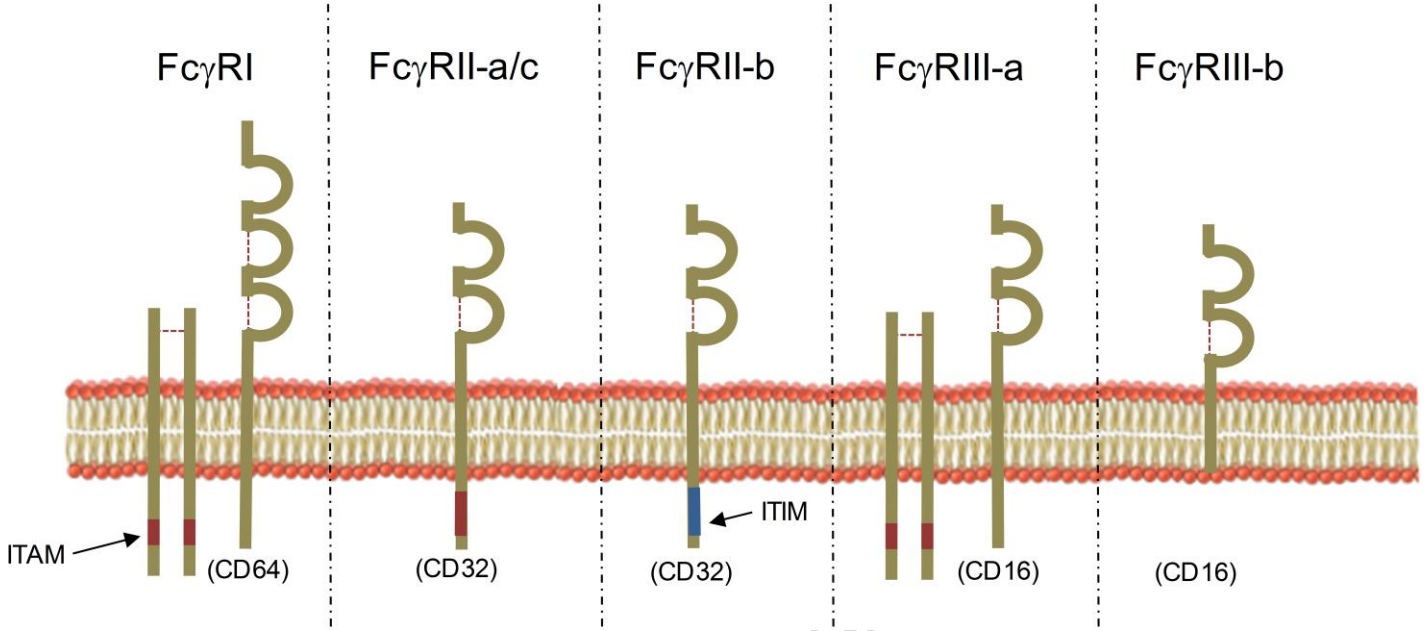

Figure 2

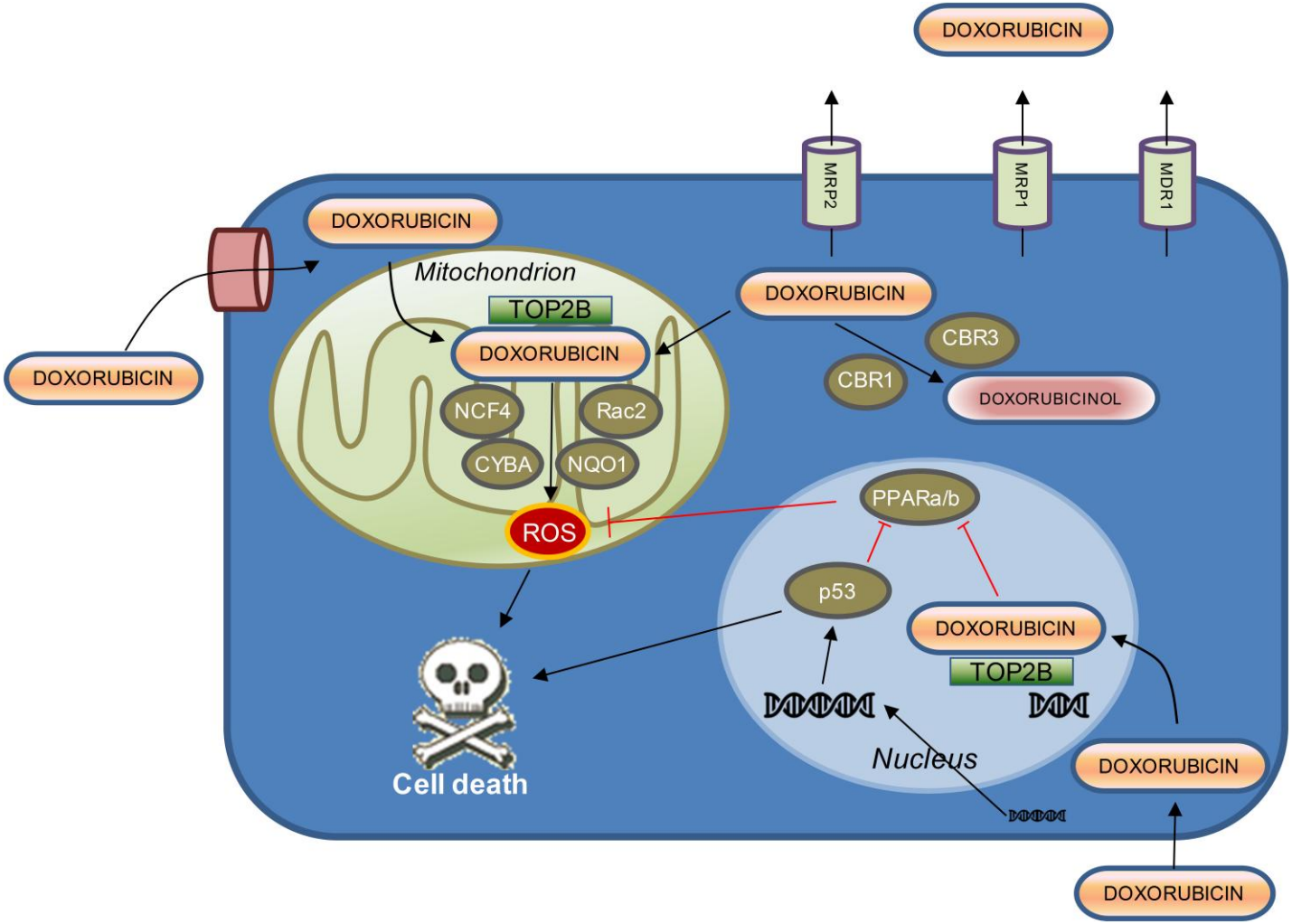

Figure 3

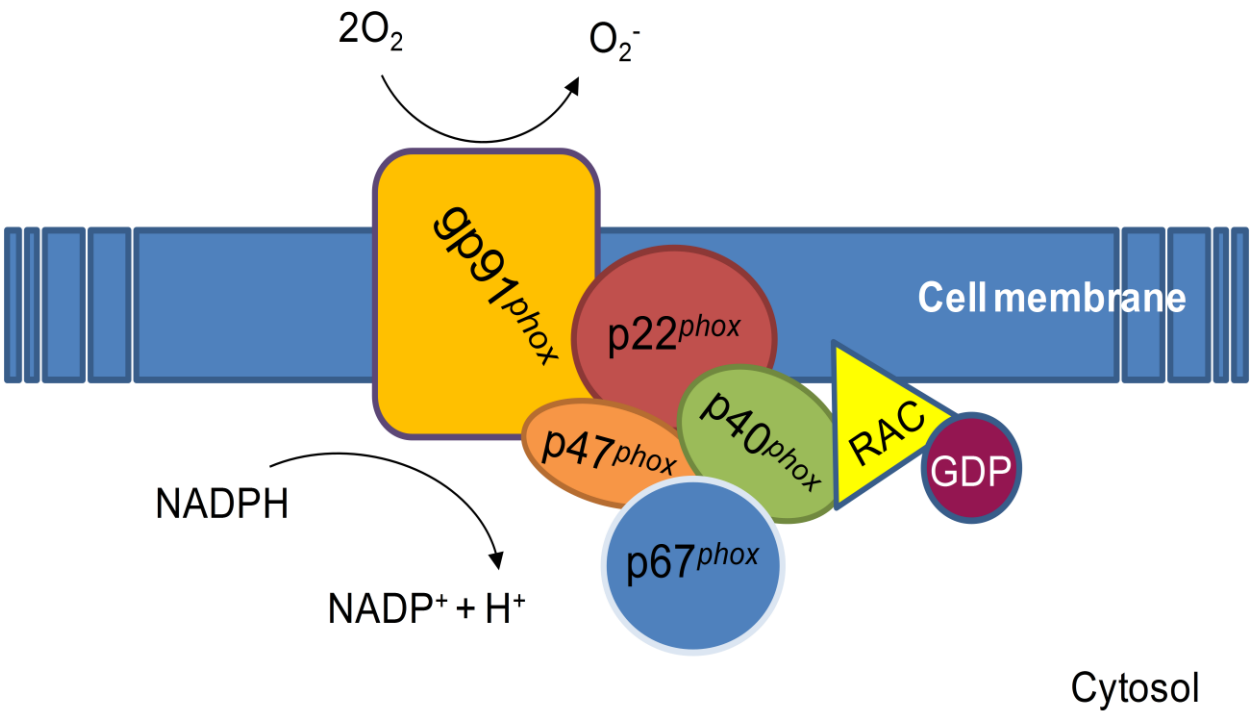

Figure 4

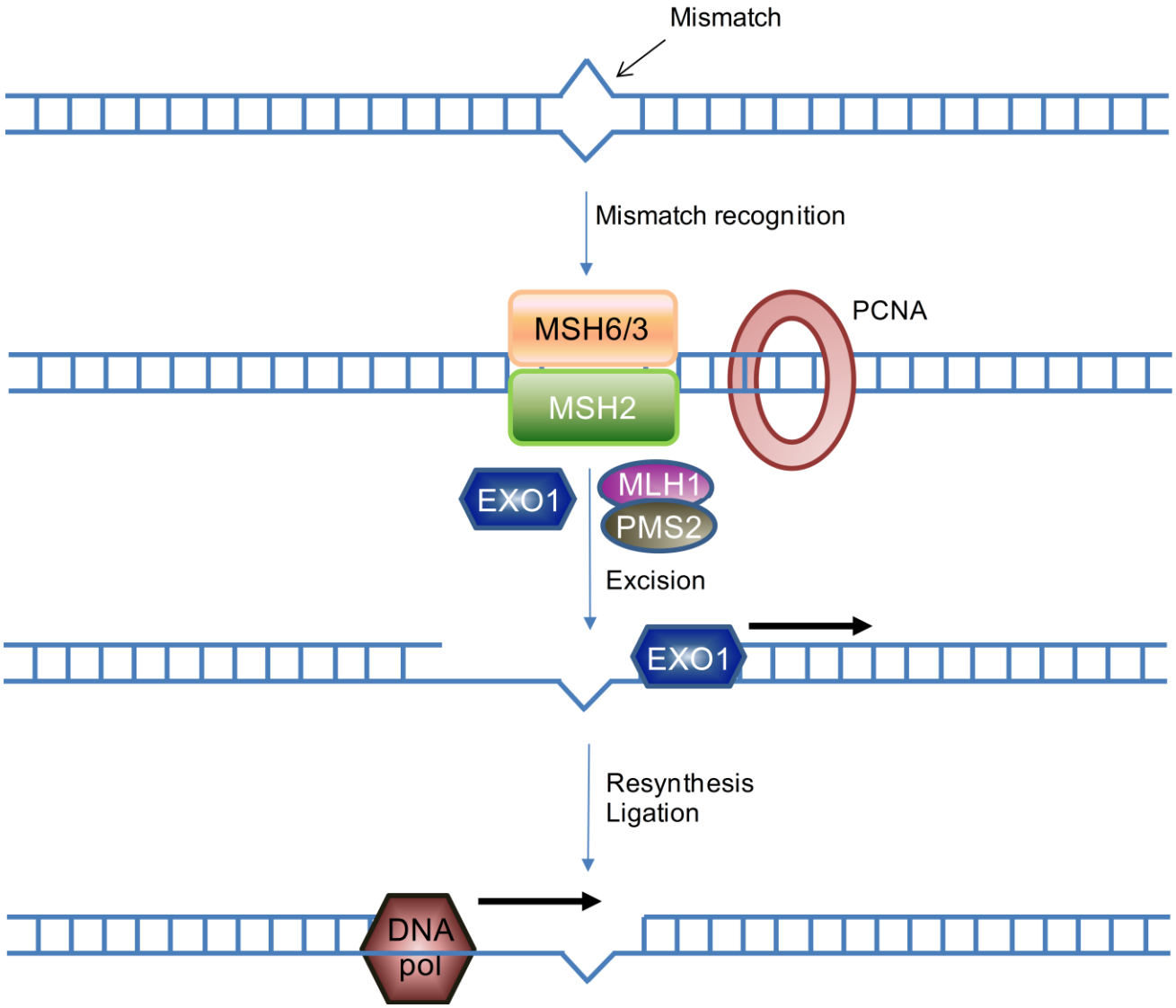

Figure 5

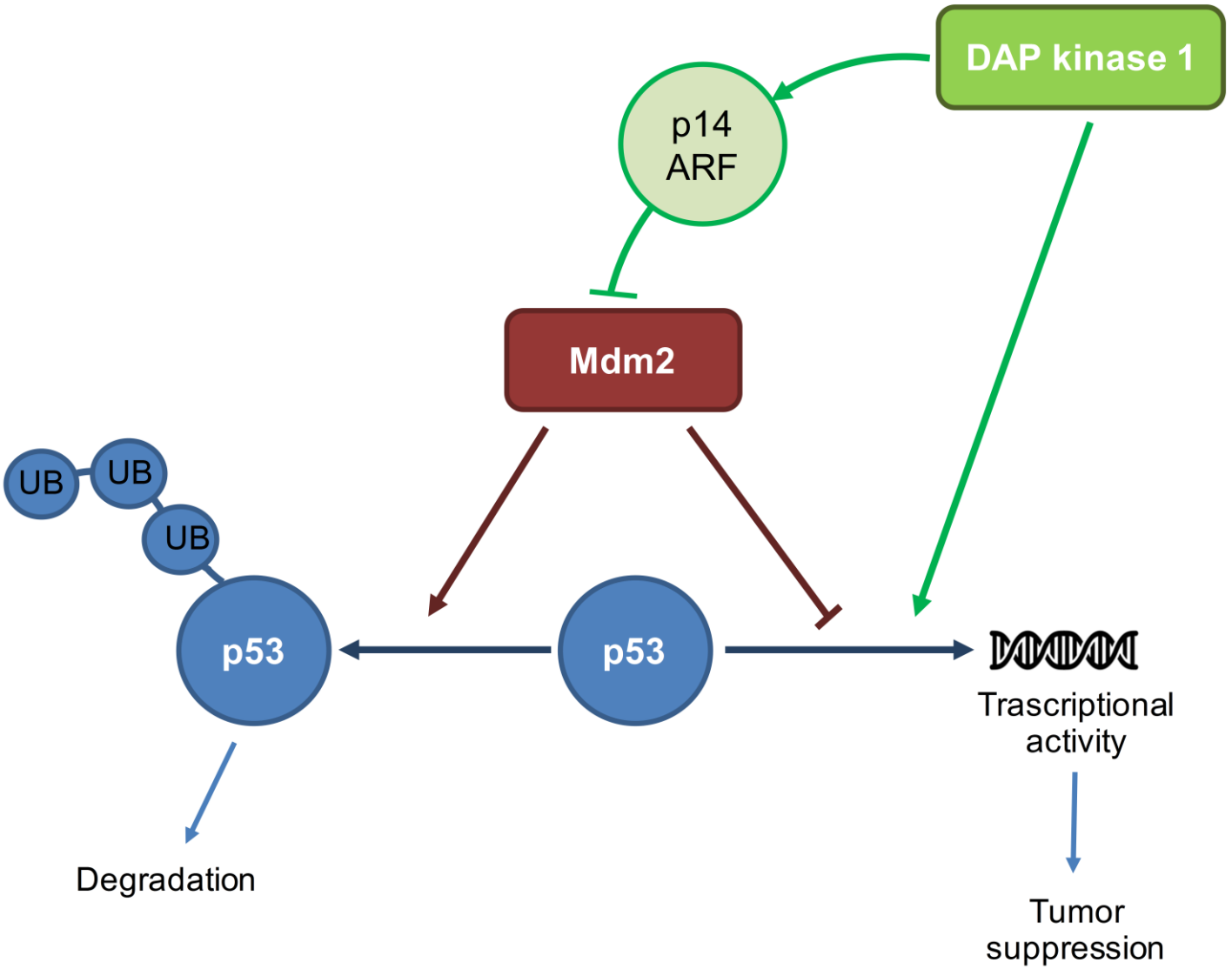

Figure 6