Molecular biomarkers of DNA damage in diffuse large-cell lymphoma—a review

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Abstract: Diffuse large B-cell lymphoma (DLBCL) is a highly aggressive form of non-Hodgkin's lymphoma with a long-term survival rate of around 60% upon standard treatment with rituximab and chemotherapy. While this marks a great improvement over the pre-rituximab/pre-biologicals era, DLBCLs remain a heterogeneous disease with marked variability in clinical outcome. Efforts have been undertaken to predict its course based on characteristics such as the activated B-cell (ABC) versus germinal centre B-cell (GCB) types, but so far none of these has had much impact on treatment and outcome, and the international prognostic index, based on clinical and basic laboratory features, remains the best risk predictor. This review focuses on 13 biomarkers that have been described in the context of diagnostic tests, clinical studies or basic research related to solid and haematological malignancies in general or DLBCL in particular. These include biomarkers of the DNA repair response such as the phosphorylated histone variant 2AX (γH2AX), p53 binding protein (53BP1), Ataxia telangiectasia mutated protein (ATM), Ataxia telangiectasia and Rad3-related protein (ATR), the DNA-dependent protein kinase (DNA-PK) and Rad51 as well as proto-oncogene products like Myc, B-cell lymphoma 2 (Bcl-2), breast cancer 1 (BRCA1) and signal transducer and activator of transcription 3 (STAT3). We have also included 8-Hydroxy-2'-desoxyguanosine (8-OHdG), a marker for oxidative stress, and discuss a link between two proteins involved in apoptosis, the proteasome activator 28γ (PA28γ) and tumour suppressor p53. Our investigation revealed a connection between almost all biomarkers, which are based on methods such as indirect immunofluorescence, fluorescence in-situ hybridization or immunohistochemistry.

Keywords: Diffuse large B-cell lymphoma (DLBCL); gamma H2AX; double-strand break (DSB); DNA damage; biomarker

Introduction

Cancer is the second most frequent cause of death worldwide according to the World Health Organisation (WHO) (1). One of the most aggressive forms of Non-Hodgkin's lymphoma, diffuse large B-cell lymphoma (DLBCL), is diagnosed in more than 50% of lymphoma patients over the age of 65 (2). As for most malignant diseases, the risk of developing DLBCL increases with age, and more than 40% of patients eventually succumb to their disease (3,4). In recent years, therapy for many cancers and leukaemia's has been fundamentally improved

based on a deeper understanding of the molecular hallmarks underlying the disease, paving the way for the concept of personalized medicine both with regard to therapy selection and response monitoring. As cancer is basically a genetic disorder, most malignant diseases arise from one sort or another of *de novo* deoxyribonucleic acid (DNA) damage, be this point mutations or chromosomal rearrangements. At the root of these lies unrepaired DNA damage, which occurs frequently under physiological conditions as the DNA is exposed to damaging agents, such as reactive oxygen species (5) or ultra-violet (UV) irradiation (6).

The most severe form of DNA damage is a DNA double-strand break (DSB). Caused by chemical or physical noxae, it leads to genomic instability (7,8) and thus increases the probability of cancerogenesis (9). Apart from cancer, DNA damage is related to diseases associated with aging such as Xeroderma pigmentosum, Cockayne's syndrome or Trichothiodystrophy (10). Some mutations in oncogenes or tumour suppressor genes, such as of the protein 53 (p53) significantly increase the incidence of cancer in patients. For example, mutations of the *p53* gene leading to inactivation of the protein can be detected in 50% of all human tumours such as for instance breast, lung, liver tumours or lymphomas, but only in a small percentage of the healthy population (11).

Currently, cancer is often diagnosed by radiological examinations or biopsies, and for a complete diagnosis consisting of grading and staging, both are usually required. As highly proliferative malignant cells run in hypermetabolic state, they can also be detected by radioactively labelled metabolic substrates in conventional scintigraphy or positron emission tomography (12,13). All these procedures are often stressful for the patient, carry the risk of injuries and adverse effects, and have a varying degree of sensitivity and specificity, which lead to diagnostic insecurity and may necessitate repeated procedures, for instance in the case of a negative tissue biopsy that is clinically highly suspicious for cancer.

A diagnostic "magic bullet" would therefore be highly desirable, as is sought for with the emerging concept of liquid biopsies, intended to replace tissue biopsies with materials from blood samples, such as soluble DNA, peripheral blood mononuclear cells (PBMC), and circulating tumour cells, for diagnosis and monitoring of malignant disease (14). Circulating tumour cells predict the probability of a poor prognosis in early stages of breast cancer and may thus give a valuable contribution to decide whether or not upon initial surgery a woman should receive adjuvant therapy. Rack *et al.* showed that patients

with at least five circulating tumour cells per 30 mL of blood have the worst prognosis and survival probability [hazard ratio (HR) for disease-free survival =4.51, 95% confidence interval (CI): 2.59 to 7.86; HR for overall survival (OS) =3.60, 95% CI: 1.56 to 8.45] and that the detection of circulating tumour cells after chemotherapy correlates with the risk of relapse (15). However, the clinical meaning of these findings is subject to clinical trials (16,17), and currently it is unclear where and how this prospective information can be used to actually improve prognosis. Technically, the liquid biopsy approach relies on biomarkers that are clinically meaningful (18) and can be easily assessed with good sensitivity and specificity (19), preferably in multiparametric screening systems.

In this review we discuss potentially relevant biomarkers for diagnosis, prognosis and prediction of therapy response in malignant diseases. We will first discuss the current genetic understanding of DLBCL and the repair systems that normally prevent a given genetic damage to manifest as malignant disease. We will then focus on DNA DSBs and biomarkers of it such as the p53 binding protein (53BP1), the histone variant 2AX (H2AX), and the kinases Ataxia telangiectasia mutated protein (ATM), Ataxia telangiectasia and Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK). This will be followed by eight other potential biomarkers associated with either cancer prevention or induction: Myc, B-cell lymphoma 2 (BCL-2), Breast cancer 1 (BRCA1), signal transducer and activator of transcription 3 (STAT3), 8-Hydroxy-2'-desoxyguanosine (8-OHdG), recombinase Rad51, proteasome activator 28γ (PA28γ), and tumour suppressor p53, which have all been suggested for monitoring and predicting the outcome of therapies in cancer patients, in particular those with DLBCL.

Diffuse large B-cell lymphoma

DLBCLs are the most common type of aggressive lymphomas with a crude incidence of 3.8 per 100,000 in Europe diagnosed in 2000 to 2002 (20). In fact, they comprise a heterogeneous entity with the common feature of large B-cells arranged in diffuse patterns. Morphologically an immunoblastic, centroblastic, and anaplastic type can be distinguished (21,22) and genetically they can be roughly categorized into the germinal centre B-cell (GCB) and the activated B-cell (ABC) subtype (22) by their genetic signatures. Their characterization by flow cytometry or immunohistochemistry relies on different

pan-B-cell antigens such as CD19, CD20 and CD22 as well as on B-cell transcription factors Paired box protein 5 (Pax5) and Organic cation transporter 2 (OCT2) (21). Untreated it is almost always lethal within a few weeks to months, but the introduction of combination chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) some 40 years ago, has by itself brought in long-term remissions and a considerable cure rate. Since then, the addition of the anti-CD20 monoclonal antibody rituximab by the end of the 1990's has further improved outcomes to response rates of 40% to over 90% and 5-year survival rates of 35% to 85%, depending on age and clinical risk group (3,23,24). The R-CHOP protocol has since been the gold standard of therapy which more aggressive therapy regimens failed to improve upon. Treatment options for patients not responding to R-CHOP or experiencing a relapse are scarce and prognosis for these patients is poor.

Patients harbouring a GCB lymphoma are more likely to survive at least 5 years upon R-CHOP therapy than those with the ABC subtype (69%±3% for GCB *vs.* 53%±5% for ABC *vs.* 60%±4% for unclassified cases; P=0.02 for GCB *vs.* ABC) (25). Nonetheless, 30% to 40% of all DLBCL patients relapse after R-CHOP therapy (3,4). Response assessment relies upon restaging by computed tomography (CT) alone or its combination with fluorodeoxyglucose positron emission tomography (FDG-PET; for the combination, PET-CT). Both are highly sensitive, and PET-CT is much more specific in distinguishing residual vital tumour tissue from fibrotic scars (26). Earlier detection of a relapse does not seem to translate into better survival and radiological follow-up frequency has recently been drastically reduced.

Hence, to improve the prognosis for patients not cured with standard R-CHOP, a better molecular understanding of DLBCL development and progression is a predominant focus of research, and significant biomarkers accessible for liquid biopsies appear to promise great gains for DLBCL patients (27).

Cellular repair mechanisms of DNA damage

For apparent reasons, in multicellular organisms cell cycle and DNA-replication are tightly controlled and a multitude of highly conserved, evolutionary ancient control and repair mechanisms usually identifies and corrects errors or submits the cell to apoptosis (28). As a result, if an error is detected, the cell cycle halts and repair mechanisms attempt to restore the original DNA sequence. Once this is successful,

the cell proceeds into the next phase of the cell cycle (29). If the DNA cannot be repaired, the cell either enters senescence (30), necroptosis (31), or apoptosis, all of which maintain genetic integrity. Only if this fails, too, the irreparable DNA damage is passed on to the next generation (32). The latter event can lead to loss of protein function or, over the course of further generations, to the accumulation of additional mutations and ultimately to the uncontrolled cell proliferation that defines cancer (29,33).

The main repair systems in mammalian cells are base excision repair, nucleotide excision repair, mismatch repair and DSB repair (28). Damage at a single nucleotide base (e.g., loss or deamination), including chemically oxidized or methylated bases such as 3-methylguanine or 8-OHdG, can be repaired by base excision repair, a mechanism present in most organisms (34). In patients with malignant tumours, high levels of 8-OHdG were found (35-37), marking the critical importance of the base excision repair system.

DNA damage involving complete nucleotides such as UV-induced pyrimidine dimers or bases modified by polycyclic aromatic compounds (10) is corrected by nucleotide excision repair. The nucleotide excision repair system is closely linked to transcription-coupled repair and DNA interstrand crosslink repair (38). A congenital defect of the nucleotide excision repair system is associated with Xeroderma pigmentosum and Trichothiodystrophy (39). Xeroderma pigmentosum patients are hypersensitive to UV irradiation and at increased risk for malignant skin neoplasms (40).

Replication errors, such as mismatched or missing bases, are corrected by mismatch repair. Major DNA damage and DSB can be corrected by homologous recombination (HRe) or non-homologous end joining (NHEJ). Defects in genes involved in HRe are found in about 50% of all epithelial ovarian cancers (41), and 15% of those affected carry germ line mutations of BRCA1 and BRCA2 (42), the genes for two proteins that interact with Rad51 in repairing DSB via HRe (43) and thus demonstrate these mechanisms' importance for preventing cancer. It is thought that during mitosis, HRe is regularly employed in the late S and G2 phases when the sister chromatids can serve as templates, whereas NHEJ is more prominently active in G1 and early S phase (44) and repairs the majority of DNA DSBs (45-47).

DNA double strand breaks and cancer

Development and repair of DNA DSB

DNA DSBs are considered the most harmful form of
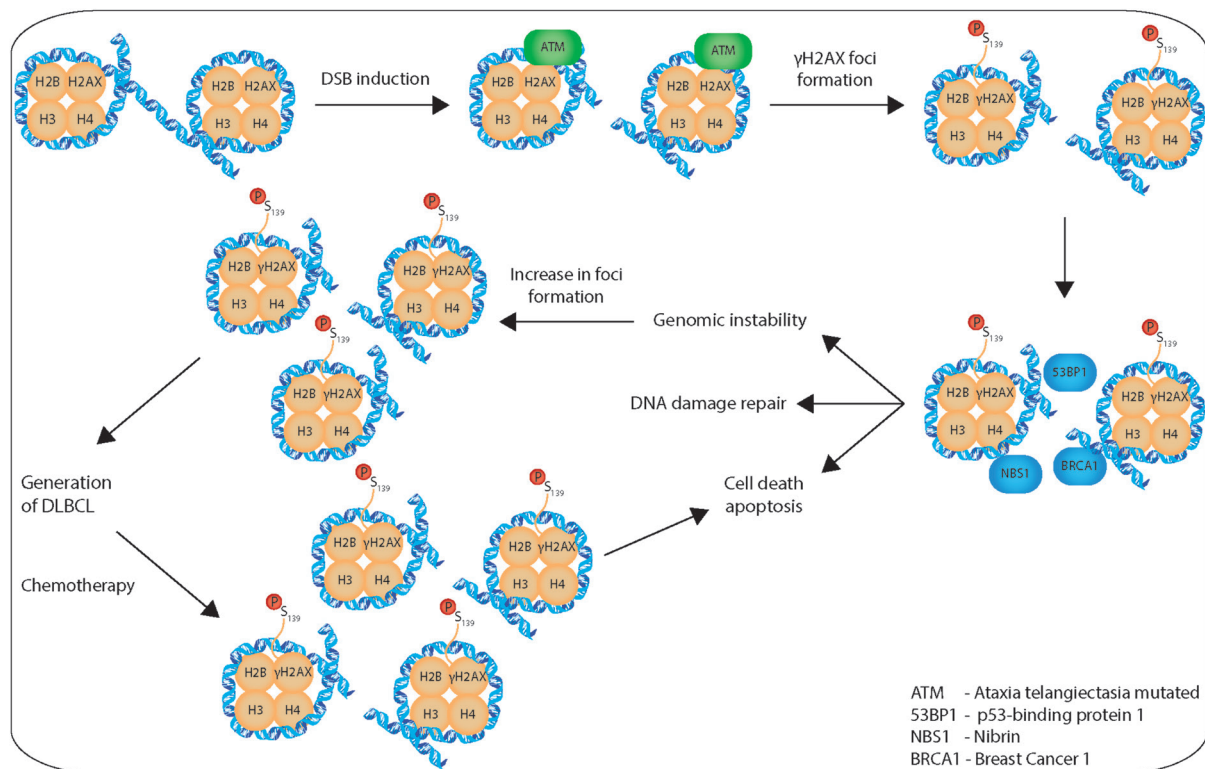
Figure 1 Schematic representation of DSB formation by DLBCL and chemotherapy treatment. The formation of DNA DSB leads to the phosphorylation of the histone variant H2AX by the kinase ATM on serine 139 (called γH2AX). Through the phosphorylation of H2AX, further DNA repair proteins such as NBS1, BRCA1 and 53BP1 are recruited to the DSB. The damaged cell can then either pass into programmed cell death, apoptosis, or the DSB is repaired. In the unfavourable event of an unrepaired DNA DSB, genomic instability can occur and increase the formation of DSB and γH2AX. This results in the formation of neoplasms such as DLBCL. Interestingly, chemotherapy treatment results into further accumulation of DSB and γH2AX, which eventually leads to apoptosis and death of the cancerous cell. DSB, double-strand break; DLBCL, diffuse large B-cell lymphoma.

DNA damage as they can promote genomic instability and increase the risk of cancer (7,33,48-51). The repair of DNA DSBs is more complex than that of single nucleotide damages because the correct anti-parallel strand is no longer available as a template (35,38,49,52). Therefore DSBs cannot be repaired through a simple ligation (49), but HRe and NHEJ need to compensate for the loss of both DNA strands (7,48,49,53). The complex machinery of histones and enzymes involved in this process also offers the methods that have been proposed to detect and quantify DNA DSBs (54). Histone modifications associated with DNA damage have attracted growing interest for early cancer detection in recent years.

The histone 2AX (H2AX) has become a widely used biomarker for DNA DSBs (51,55-57). Upon the occurrence of a DSB, H2AX is phosphorylated at serin 139 (then termed γH2AX) by members of the phosphoinoside-3 kinase (PI-3K) family and binds to the damaged DNA (*Figure 1*) (55,56). The formation of γH2AX is easily detected using antibodies against the phosphorylated variant, which can be localized as discrete nuclear foci (57-59).

The phosphorylated histone 2AX variant is associated with interleukin 1α, which is involved in inflammation, microbial diseases and cancer and influences tumours by promoting proliferation, the expression of chemokines, adhesion molecules, and pro-survival factors. During stress such as UV irradiation, interleukin 1α is secreted by necrosis or pyroptosis (60) and co-localizes with γH2AX and histone deacetylase-1 at sites of DNA damage. The function of this nuclear localization is not known so far, but localization with biomarkers such as 53BP1 (61-64), BRCA1 (65,66)

and Rad51 (66) was also observed for γH2AX. Those co-localizations indicated an association of γH2AX with DNA damage repair, cell cycle progression and HRe.

Increased γH2AX foci formation has been observed in a wide variety of tumours and meanwhile has been investigated in more than 35 clinical studies [reviews in (67,68)]. Its prognostic relevance has been shown for different tumours such as triple negative breast cancer (P=0.01; HR =4.06, 95% CI: 1.40–11.77) (69). Incubation of PBMCs with different chemotherapeutic drugs such as etoposide, rapamycin (57) or doxorubicin (70) as well as irradiation (51,71-74) induces γH2AX formation (51,57,70,75). Hence it has been suggested that cytotoxic chemotherapy may further increase γH2AX foci formation in cancer patients, who already experience more DNA DSBs than healthy donors (*Figure 1*). Interestingly, γH2AX foci also appear to increase consistently with exercise (P=0.04 to <0.001) as observed with runs ranging from 5 to 42 km (P<0.001) (76).

In addition to the promising application possibilities, there are also controversial concerns about the use of H2AX as a biomarker. These are based primarily on the high heterogeneity of tumour diseases. It is also debatable whether *ex vivo* analyses really have the same significance as *in vivo* analyses. There is also evidence that results of γH2AX foci assay depends on the experimental settings (77). High heterogeneity would also be expected in *in vivo* samples due to uncontrollable factors such as nutritional intake, oxygen supply or tumour microenvironment. However, a study by Rassamegevanon *et al.* demonstrated a high grade of heterogenetic results when human head and neck squamous cell carcinoma models were treated with ionising radiation *ex vivo*, compared to *in vivo*. This mean that the transfer of a tumour into a new microenvironment could increase the degree of heterogeneity in H2AX foci (77). Whether this is also the case for liquid biopsies such as the isolation of PBMCs is subject to ongoing debate.

Regarding DLBCL in particular, Derenzini *et al.* found phosphorylated H2AX in 47% of patients where it appeared to predict 5-year OS, which was 41% in γH2AX positive as opposed to 70% in γH2AX negative patients (P<0.01) (68). While these data need to be verified in larger cohorts, they suggest a potential prognostic value of this parameter and might in addition point at a novel therapeutic target as the authors suggest.

Involvement of DNA kinases

Phosphorylation of H2AX can be catalysed by three kinases:

the serine protein kinase ATM, the phosphatidylinositol 3-kinase-related kinase DNA-PK, and the serine/threonine protein kinase ATR (78,79). Defects in ATM increase cancer incidence in patients and can lead to the eponymous Ataxia telangiectasia syndrome which is associated with increased radiation sensitivity (80-82). Upon DSB induction, ATM phosphorylates downstream targets such as p53, mouse double minute 2 homolog (Mdm2), checkpoint kinase 1 and 2 (Chk1 and Chk2), BRCA1 and nibrin next to H2AX and thus has a variety of influences for instance on apoptosis, cell cycle checkpoints and DNA repair (33,48,83). The exact function of ATM-mediated phosphorylation remains unclear (79,84). However, γH2AX foci formation is strongly reduced in cells which lack DNA-PK and reduced ATM levels (66).

In DLBCL patients, ATM mutations have been found to be associated with inactivation of the tumour suppressor gene *ARF* and *tumor protein P53* (*TP53*). Missense variants of ATM have been discovered and are of unclear functionality. Nonetheless, it was suggested that mutations in ATM and inactivation of the *ARF-TP53* pathway may promote DLBCL (85). As loss of its function appears to lead to genomic instability, cancer susceptibility and profound sensitivity to ionising radiation (86,87), ATM is a potential target for cancer therapy. On that account, several ATM inhibitors are of interest such as Wortmannin (88), KU-55933 (89) or the most potent ATM-inhibitor KU-559403 (90), although none of these is currently in clinical development (*Table 1*) (87). Among these, KU-559403 stands out for its improved bioavailability and solubility (90). In a small clinical study, 55% of DLBCL patients had a mutation of ATM in addition to TP53 (P=0.015, n=9), which suggests a non-random connection (85). The ATM kinase can be detected by immunoblotting and has been shown to be an additional diagnostic tool for Ataxia Telangiectasia besides the colony survival assay (100).

Defects of ATR, too, increase radiation sensitivity and reduce monitoring of cell cycle checkpoints. This kinase phosphorylates proteins such as H2AX (65), Chk1 (93) and BRCA1 (147) and helps to activate the cellular tumour antigen p53 (148). Therefore, ATM and ATR play a central role in the cellular response to radiation, and their functions overlap. They influence each other through mutual expression regulation (131,132,149) and phosphorylation (150,151). Potent inhibitors of ATR are NU6027 (152), VE-821 (133) or AZD6738 (87). The latter is reportedly being examined in a clinical study (ClinicalTrials.gov: NCT02223923),

Table 1 Potential biomarkers for monitoring disease outcome of DLBCL

Potential Biomarker	Target structure	Detection Method(s)	Clinical relevance	ClinicalTrails.gov Studies	Additional References
53BP1	Protein, DNA	IIF, FISH	Poor diagnostic value in MS (63), correlation with molecular grouping in triple breast cancer (91), poor prognosis in triple negative breast cancer (69), foci formation after irradiation in mesenchymal stem cells (92)	6	(63,92,93)
8-OHdG	Protein	IIF, HPLC, ELISA, IHC	Quantification of oxidative stress (94-97), prognostic marker for lymphoma (98)	32	(97,99)
ATM	Protein	IIF, WB	Mutation contribute to DLBCL (85)	103	(100-102)
ATR	Protein	IIF	Inhibitor used in clinical studies (87)	36	(103)
BCL-2	DNA, Protein	FISH, IHC	Risk-stratify of DLBCL patients (104), predict recurrence of prostate cancer after radical prostatectomy (105), prognostic marker in breast cancer (106), inferior survival of DLBCL (107), phase-II study of inhibitor (108)	219	(104)
BRCA1	Protein, DNA	WB, FISH, IIF	Mutations in BRCA1 also indicated high occurrence of γH2AX in breast cancer (69), promoter hypermethylation associated with more aggressive breast cancer profile (91), cisplatin chemotherapy high activity in breast cancer with BRCA1 mutation (109,110), BRCA1 level determines OS in lung carcinomas (111), higher breast cancer risk for females with BRCA1 mutation (112,113)	334	(114,115)
Myc	DNA, Protein	FISH, IIF, IHC, qPCR	Poor prognosis for DLBCL (107,116), risk-stratify of DLBCL patients (104,117), prognostic value for OS in DLBCL (118), Myc rearrangement strongly adverse prognostic factor (119), pSTAT3 expression associated with Myc (120)	99	(104,114, 121-123)
DNA DSB (γH2AX)	Protein	IIF, FISH, WB, FACS	Poor diagnostic value in MS (63), poor prognosis in triple negative breast cancer (69), γH2AX increase during exercise (76), increased in CTC and PBMCs after treatment with PARP-inhibitor (124), prediction of OS in DLBCL (68), quantitative biomarker for low-dose radiation (72), increased foci number in chronic lymphocytic leukemia (125), foci formation after irradiation in mesenchymal stem cells (92) induction after treatment with aza-cytosine nucleosides (126) and radiotherapy (127) implies integral body dose (128), γH2AX declines with increased age (129), biomarker for DNA damage in TP53 mutated high-risk endometrial cancer (130)	37	(55,92, 131-133)
DNA-PK	Protein	WB, IIF	Cytotoxicity induction and inhibition of chronic lymphocytic leukaemia survival signals through DNA-PK inhibitors (134), high-risk endometrial cancer deficient for DNA damage response biomarkers (130)	7	(102,132)

Table 1 *(continued)*

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Potential Biomarker	Target structure	Detection Method(s)	Clinical relevance	ClinicalTrails.gov Studies	Additional References
p53	Protein, DNA	IHC, HPLC, FISH, DNA Microarray	Predict recurrence of prostate cancer after radical prostatectomy (105), associated with increased tumour size of breast cancer patients (size expressed as binary variable) (106), higher amount of DNA damage in TP53 mutated high-risk endometrial cancer (130), Predicted output of DLBCL (135), mutation of TP53 correlate with poor OS in DLBCL (117,136)	362	(114,117, 136,137)
PA28γ	Protein	WB, ELISA	Used in one clinical study depending neoplasms (138)	No study found	(139,140)
Rad51	Protein	IIF, WB	Foci formation after prolongs irradiation in mesenchymal stem cells (92), trend for improved survival in high-risk endometrial cancer and positive correlation with γH2AX (130), low Rad51 scores correlated with high histological grade of breast cancer and predict chemotherapy response (141), co-localization with BRCA1 (66) and 53BP1 (130)	44	(142-144)
STAT3	Protein	IHC, DNA Microarray	Clinical trial target for DLBCL (173), pYSTAT3 predicted outcome of DLBCL and is associated with poor OS (135,145,146)	64	(146)

Biomarkers are listed with target structure and detection methods. Studies on ClinicalTrail.gov were found by searching for Cancer and the specific biomarker. ELISA, enzyme-linked Immunosorbent Assay; FACS, fluorescence-activated cell scanning; FISH, fluorescent *in situ* hybridisation; HPLC, high performance liquid chromatography; IHC, immunohistochemistry; IIF, indirect immunofluorescence; qPCR, quantitative Polymerase chain reaction; WB, Western Blot (Immunoblotting).

which is currently suspended, however (*Table 1*) (153). Biomarkers to monitor the effects of ATR and/or ATM inhibitors have not been established. Suggestions include p53 and activated oncogenes such as Myc, pTP53, γH2AX, as well as ATM or phosphorylated ATM itself (87). Immunostaining of ATR was performed by Jazayeri *et al.* and showed that ATM and ATR work in the same signalling pathway upon irradiation, with ATM functioning upstream of ATR (*Table 1*) (103). Moreover, co-localization of ATM and γH2AX foci at sites of DSBs has been shown in Hela cells (101).

DNA-PK is an enzyme complex consisting of the three components Ku80, Ku70, and the catalytic subunit DNA-PKcs (DNA-dependent protein kinase catalytic subunit), and is involved in NHEJ. This complex only assembles immediately on the DNA to be repaired. Ku70/Ku80 initially binds as an annular heterodimer to the broken DNA double strand ends. Subsequently DNA-PKcs joins this heterodimer, leads to a change in conformation and thus activates the catalytic subunit (45,154,155). Two DNA-PK complexes are able to mediate joining of DNA at opposite ends, prevent inadvertent loop formation and reassemble the two strands (156). Knockout mouse embryonic fibroblast cells lacking this complex were more susceptible to damage from ionizing irradiation and showed slower DSB repair with prolonged cell cycle arrest. Lack of DNA-PK seemed to affect overall DNA damage response only little, but to disrupt severely the repair of DSB (102). Formation of γH2AX foci upon irradiation was similar in DNA-PKcs deficient and wild type mouse cells, but could neither be detected in ATM-deficient cells nor after exposure to high doses of the phosphatidylinositol 3-kinase inhibitor Wortmannin. In conclusion, in the absence of ATM, DNA-PK appears to maintain a low level of H2AX phosphorylation (78). Hence it is probably directly involved in H2AX phosphorylation and foci formation at the site of DSBs. DNA-PK foci themselves can be detected by indirect immunofluorescence (IIF) (132) and offer another marker for DNA-DSBs amenable to automated immunofluorescence imaging (*Table 1*).

53BP1 as diagnostic biomarker for DSB repair

The DNA damage checkpoint protein p53 binding protein 1 (53BP1) accumulates rapidly with γH2AX at the site of DNA DSBs (61,62) and is itself a substrate for ATM-dependant phosphorylation. The protein appears to become physiologically phosphorylated to some low degree, but hyperphosphorylation was detected only upon ionizing irradiation (62). In DLBCL a single copy loss in the *53BP1* locus on chromosome 15q15 was found (157), and this haploinsufficiency may be a predisposing event in some DLBCL. 53BP1, too, plays a crucial role in DSB repair (61,62,157). H2AX is not necessary to recruit 53BP1, but is needed for maintaining and increasing 53BP1 levels at a DSB site (158). Upon a noxa, the protein was shown to co-localize with γH2AX (61-64) and BRCA1 (65). Moreover, quantitative γH2AX levels correlated with 53BP1 (P=0.053) and BRCA1 (P=0.011) in breast cancer cell lines (69). In triple negative breast cancer, 53BP1 protein expression correlated with molecular grouping of unmethylated BRCA1 promoter (53% of unmethylated *vs.* 27.8% of methylated cancers, P=0.045) (91). As opposed to these malignant diseases, in multiple sclerosis (MS) neither γH2AX nor 53BP1 foci analysis differed from healthy donors (for both biomarkers P=0.6) (63). However, 53BP1 is involved in DNA damage-response pathways (159) and enhances p53-dependent transcription (160). Since 53BP1 is considered a marker for the repair of DNA DSB, its use together with γH2AX, which is a marker for the early detection of DSB (*Table 1*), would be advisable.

Detection of DNA DSB and their application as diagnostic biomarker

Various alternative methods are employed to detect DNA DSB. The classical method is the single cell gel electrophoresis or comet assay (so named after the migration pattern of DNA) of Östling and Johansson (161). But 100 times greater sensitivity can be achieved by flow cytometry (162). Using γH2AX as a common biomarker for DNA damage has since become popular. Immunoblots are conventionally used to detect the histone in total protein lysates. However, besides its lower sensitivity compared to immunofluorescence (57), immunoblotting is time-consuming and ill-suited for larger sample numbers in a clinical setting (7,163). To counter these drawbacks, fully automated IIF platforms have been developed and adapted specifically for the analysis of human lymphocytes (also summarized in *Table 1*) (51,57,70,164-173). Equipped with a computerized image analysis, an automated IIF platform demonstrated superior sensitivity compared to fluorescence microscopy, flow cytometry or immunoblotting on PBMC treated with etoposide (174). Experiments showed a dose-dependent increase of the γH2AX damage response as a consequence to cytotoxic treatment (51,57). The advantage of automated IIF is the detection of individual foci per cell (count data), nucleus size (diameter) and fluorescence intensity (57). As opposed to flow cytometry and manual fluorescence microscopy, automated microscopy allows for the capture of multiple z-planes in micrometre distances and subsequent three-dimensional analysis (64). In addition, several fluorescence colours can be integrated into one multiplexing analysis, limiting the number of cell structures to be evaluated simultaneously only by the available fluorescence dyes. In our group, we have analysed the influence of cryopreservation of human PBMCs with this technique. Surprisingly in the light of cell therapies using the same cryoconservation technology, we detected a significant decrease in γH2AX levels after cryopreservation (175).

Fluorescent *in situ* hybridization (FISH) is a widely applied and highly sensitive method for detecting specific nucleic acid sequences through the specific binding of labelled DNA or RNA probes (114,176). Combining γH2AX fluorescence microscopy with FISH offers a way to characterize the DNA actually affected by detected DSBs.

Nakamura *et al.* showed that telomere damage highly contributed to overall endogenous DNA double-strand damages in tumour cells (177). Telomere-specific FISH probes were used to differentiate telomeric from extra-telomeric DNA damage. Combined analysis of γH2AX foci and FISH allowed to discriminate DNA double-strand ends from DSBs at chromosomal ends, e.g., within telomeres. This study found that γH2AX foci were directly related to the extent of telomere damage and inversely related to telomerase activity (177).

Brugat *et al.* used FISH for the detection of complex chromosomal aberrations in chronic lymphocytic leukaemia cells known for telomere dysfunction (125) and showed that critically short telomeres activate the same response as DSB, characterized by foci of Telomeric repeat-binding factor 2 and γH2AX and/or 53BP1 as well as proteins of the NHEJ repair system. By analysing telomere and other frequent chromosomal deletions in metaphase chronic lymphocytic leukaemia cells, they demonstrated an association of short telomeres with genomic instability (125).

FISH was also successfully applied to investigate the role

of H2AX in B-lymphocytes during the recombinatorial switch in immunoglobulin heavy chain class (178,179). Using FISH probes targeting different regions of the heavy chain genes, Franco *et al.* and Yin *et al.* proposed a general role of the DSB response in promoting end joining and preventing chromosomal breaks or translocations through chromatin modifications performed by H2AX (178,179).

As mentioned earlier, while DSBs may cause cells to become genetically instable and then cancerous, the vast majority will first halt the cell cycle and then lead to apoptotic cell death (*Figure 1*). Some cytotoxins utilize the lethal effect of DSBs by either inducing them (etoposide) or by provoking malfunctions of critical enzymes such as topoisomerase II (anthracyclines, irinotecan) (57,70). The DNA-DSB response factors γH2AX, 53BP1, ATR, ATM, and DNA-PK could therefore be used to monitor cancer treatment and possibly predict its outcome from PBMC.

The proto-oncogenes Myc and BCL-2 as diagnostic markers

To get a broader picture in the development of DLBCL, other biomarkers should be considered. The proto-oncogene Myc and the anti-apoptotic protein BCL-2 are both involved in the regulation of apoptosis. The overexpression of Myc lowers the level of BCL-2 and promotes apoptosis (180). In most oncological cases, increased expression of Myc however results in overexpression of BCL-2, which favours anti-apoptosis in cancer cells (181). Therefore, Myc and BCL-2 are key players in inducing oncological diseases.

Lymphomas with rearrangements of the proto-oncogene Myc (also known as c-Myc) carry a poor risk even with CHOP or R-CHOP therapy (182). There are two controversially discussed types of FISH probes for Myc rearrangements, break-apart probes and dual-fusion probes (119,182), of whom the break-apart probes appear more efficient (183). Some DLBCL patients show Myc overexpression without gene alterations: existing data suggest that only one third of patients with Myc overexpression actually carry a translocation (104,121), while other authors maintain that these be false positives and should be re-evaluated by FISH screening (*Table 1*) (122).

Myc was identified as a biomarker for DLBCL in a meta-analysis comprising 4662 cases. The HR of patients with c-Myc abnormalities compared to DLBCL patients without, calculated and analysed from 19 different studies, was situated at 2.22 (95% CI, 1.89 to 2.61) (184). The majority of studies in this meta-analysis found Myc predictive for the outcome of DLBCL (184).

The oncogenic effect of Myc depends rather on mutations influencing its quantitative expression than on qualitative alterations of its fundamental functions (185). DNA breaks within the *myc* gen can be detected by quantitative PCR, but no correlation between Myc DNA breaks and high mRNA levels was found. Immunohistochemistry (IHC) Myc staining correlates with *myc* transcript numbers. For a precise detection of several Myc mutations, multiple or multiplex Polymerase chain reaction (PCR) would be necessary (122). But the optimal method for the detection of *myc* expression still seems to be controversial.

It is suggested that Myc is associated with nearly half of all human oncological diseases (185). In ovarian cancer, patients expressing high levels of Myc mRNA lived shorter with earlier relapses (186). Along the same line, high *myc* expression indicated poor overall survival in DLBCL (123).

In addition to these findings, Johnson *et al.* showed that BCL-2 determines the outcome of Myc-positive DLBCL. The so-called double-hit (Myc-positive and BCL-2-positive) phenotype has been confirmed as a potent predictor of poor risk (104). Detection of BCL-2 and Myc protein expression by IHC is cheaper, faster, and equally reproducible compared to the detection of Myc translocations by FISH (104). Molecular hallmarks such as BCL-2 or the ABC type deteriorate the already poor outcome, as well as R-CHOP treatment in elderly patients (187-190). DLBCL patients are BCL-2 positive in 66% of cases and responded better to R-CHOP therapy than to a therapy without rituximab. Those patients also indicated a better overall survival (67%±9% versus 48%±11%, P=0.004) than patients positive for BLC-2 treated with CHOP alone (189). In addition, the frequency of Myc positive cases increases from 35% to 62% when γH2AX negative cases are compared to positive patients (P=0.02) (68), making it a more likely candidate then BCL-2.

BRCA1 as biomarker for breast cancer and lymphoma

Mutations in the tumour suppressor gene BRCA1 (also termed breast cancer susceptibility gene 1) are predictive for highly increased risks of breast and ovarian cancer, and about 5% of all breast cancer cases can be attributed to BRCA1 mutations. However, its tumour suppressor function is ubiquitous and failure of it is also involved in

lymphoma (92). The median survival in a low BRCA1 group of non-small-cell lung cancer patients was 11 month (95% CI: 1.1 to 20.9) and two-year survival rate was 41.2% compared to the high BRCA1 group with same median survival (95% CI: 8.2 to 13.8), but a two-year survival rate of 0% (94). BRCA1 is associated with genotoxic stress and γ-irradiation (95,96). Increased γH2AX levels were detected in BRCA1-mutated breast cancer cell lines (P=0.011) (69). Phosphorylation of H2AX has been shown to be a recruitment signal for BRCA1 (66). Upon DNA damage, γH2AX and BRCA1 form a biochemical complex within the chromatin fraction (95) and are recruited to damage sites even before the repair factors Rad50 and Rad51 (66), which appear to form a complex together with 53BP1 (65,66). Knocking down of BRCA1 by antisense morpholino oligonucleotides substantially reduced H2AX ubiquitination while increasing its phosphorylation (95). It has therefore been suggested that BRCA1 is part of a late phase of DNA repair, and one function of it is to attenuate and eventually terminate the early response marked by γH2AX (95).

As BRCA1 is detectable by immunoblotting and IIF (66,95), it may easily be included in a marker panel for DLBCL, too (*Table 1*).

STAT3 and its importance in cancer monitoring

The signal transducer and activator of transcription 3 (STAT3) is a transcription factor regulating genes involved in proliferation, angiogenesis and suppression of apoptosis as well as cell-cycle control, the latter by regulating expression of Myc and cyclin D1 (97). These properties give it the potential to become a strong oncogene if mutated or dysregulated (98). In DLBCL, STAT3 was demonstrated to promote apoptotic resistance in the aggressive ABC subtype (99). Higher levels of STAT3 mRNA were found in ABC compared to GCB DLBCL (105), and high nuclear STAT3 expression in DLBCL tissue samples was associated with poor overall survival (P=0.005) (106). However, not all ABC-type DLBCLs displayed high STAT3 mRNA.

This led to the following conclusions:

❖ Molecularly different subsets exist within ABC-type DLBCL;
❖ Differences between STAT3-high and STAT3-low ABC subtypes were suggested to be dependent on expression of anti-apoptotic BCL family members
  ◆ STAT3 high cases were BCL-2 low/MCL1;
  ◆ STAT3 low cases were BCL-2 high/MCL1 (105).
❖ STAT3 is associated with Myc and BCL-2

expression;
❖ Phosphorylated STAT3 levels correlated with poor outcome in DLBCL patients (107).

DLBCL patients positive for pSTAT3 showed a 5-year overall survival of 39.9%, compared to 56.2% in pSTAT3-negative patients (P=0.016) (107). Phosphorylation of tyrosine 705 (pY-STAT3) as observed in the majority led to a significantly better outcome after high dose chemotherapy and autologous stem cell transplantation than patients without this phosphorylated variant with a 5-year event free survival of 92.9% (95% CI: 79.4–100%) versus 47.4% (95% CI: 24.5–70.4%, log-rank P=0.006). A 5-year OS rate of 100% was detected for patients positive for pY-STAT3 compared to an OS rate of 57.9% in patients without tyrosine phosphorylation (P=0.007). Moreover, DLBCL patients harbouring no pY-STAT3, but additionally were positive for p53 expression indicated the worst 5-year OS rate of 40% (P=0.004) (108).

An association between STAT3 and γH2AX was identified in STAT3 knock-out mouse embryonic fibroblasts. When these were treated with etoposide, H2AX was still phosphorylated, but at reduced levels in the STAT3 knock-out compared to normal cells. The reduced formation of γH2AX correlated with ATM activity, and STAT3 appears likely to be important for efficient DNA DSB repair mediated through ATM and γH2AX (109). This makes STAT3 another potential candidate biomarker to predict therapy outcome in DLBCL.

8-Hydroxy-2'-desoxyguanosine

8-Hydroxy-2'-desoxyguanosine (8-OHdG) is a reaction product of DNA oxygenation, which in turn is strongly associated with DNA damage and cancer (110,111). Morning urine concentration of 8-OHdG is used as a biomarker for oxidative stress. Experiments on rats have shown that both 8-OHdG level and frequency of DNA damage increase during physiological aging (112). Quantifying 8-OHdG, however, is not trivial. Techniques such as single-cell gel electrophoresis or comet assay are still used (113), but most often its concentration in liquid samples is quantified by high pressure liquid chromatography (HPLC) (36). Recently, a cell-based automated immunofluorescence method has been established that detected foci after treatment of cells with menadione and H_2O_2, promising higher efficacy than previous approaches (115). Antibody labelling techniques such as ELISA or IHC are used to detect 8-OHdG. Each

method has its advantages and disadvantages and also the type of sample (blood, urine, plasma) should be considered in the detection of 8-OHdG, which has already been discussed elsewhere (116).

Clinically, increased urinary 8-OHdG has been shown in acute myeloid leukaemia patients during chemotherapy ($P<0.05$), but also in adult T cell leukaemia/lymphoma compared to healthy controls ($P<0.05$) (117). Similar results could be obtained in patients with prostate or bladder cancer compared to healthy donors with significant increased urinary DNA lesions (bladder cancer $P<0.001$; prostate cancer $P=0.021$) (118). These observations suggest that 8-OHdG might be a predictive marker in DLBCL, too (*Table 1*). Indeed, one study observed a higher 3-year progression-free survival rate in patients with low compared to high 8-OHdG levels ($P=0.032$, progression-free survival 77.8% *vs.* 28.6%) (120).

Rad51, a connection between NHEJ and HRe repair

The two major pathways of DNA DSB repair, NHEJ and HRe, are essential for maintaining genomic integrity. Within these two, NHEJ appears to be triggered rather by γH2AX, while HRe is mediated by Rad51, a recombinase critically involved in searching for the intact homologous DNA duplex, DNA pairing and strand exchange.

Rad51 is a homologue of the *Escherichia coli* RecA and a target for factors such as p53, BRCA1 and BRCA2 which mediate DNA repair, transcription, replication and cell-cycle progression (124,126). Its baseline levels are low and increase markedly upon ionizing irradiation (127,165). This up-regulation depends primarily on a functional HRe repair pathway and Rad53-dependent DNA-damage signalling, less so on the primary DNA damage response as indicated by H2AX activation (124). During DNA DSB introduction, Rad51 foci can be detected at damage sites by immunofluorescence microscopy. Cell lines not capable of HRe due to lack of BRCA1 or BRCA2 are unable to generate Rad51 foci (127). In experiments on mesenchymal stem cells, irradiation induced Rad51 foci only in Ki67-positive, but not in Ki67-negative cells, thus distinguishing proliferating from quiescent cells. Foci formation of γH2AX, by contrast, were induced by radiation in a dose-dependent manner irrespective of proliferation or quiescence (128). In IMR90 human lung fibroblast cells, Rad51 and γH2AX foci co-localized upon irradiation within two to six hours, suggesting that the HRe and NHEJ pathways are co-ordinated and their repair proteins are recruited to specific chromatin sections (66). Furthermore, a positive correlation of Rad51 and γH2AX was observed in endometrial cancer (R =0.318; $P=0.001$). In 27% of these cancers, loss of nuclear Rad51 was described with γH2AX foci still detectable, suggesting a defect in HRe but not NHEJ (129,165).

Clinically, low Rad51 levels in patients with sporadic primary breast cancer were associated with high histological grade ($P=0.031$) and high baseline Ki67, a marker for proliferation ($P=0.005$). In the same study, complete responses were observed in 33% of cancers with low Rad51 versus 3% in the comparison group ($P=0.011$), emphasizing the dual role of genetic instability for tumorigenesis and for cytotoxic sensitivity (127).

In summary, these results make Rad51 a biomarker for the HRe repair system and a candidate prognostic biomarker in human cancer in general (127-130). *In vitro* Rad51 inhibition in DT40 chicken B lymphocytes led to cell cycle arrest in G2/S phase and subsequent cell death (134), suggesting a vague relationship between Rad51 and B-cell lymphomas. In the context of lymphoma, however, Rad51 has not yet been addressed clinically (*Table 1*).

PA28γ and p53 as a link to apoptosis and repair pathways

Alterations in *TP53* (p53 gene) were found in many tumours. *TP53* mutations are present in approximately 21% of all DLBCL patients, and despite differences in the exact figures among studies, it is associated with poorer survival (135-137). In a small sample of 23 DLBCL patients, Young *et al*. found a trend towards shorter survival in the presence of *TP53* mutations for patients with GCB, but not with ABC subtype ($P=0.057$ and 0.96, respectively) (137). As both subtypes carry *TP53* mutations equally often, this result was to be questioned, and looking at close to 400 patients, Xu-Monette *et al*. confirmed a significantly worse prognosis in *TP53*-mutated versus wild-type patients. This applied to both the GCB and ABC subtype with hazard ratios of 0.46 and 0.52, respectively, for overall survival and of 0.45 and 0.57, respectively, for progression-free survival (136).

The most hazardous mutations occur in the DNA-binding domain of *TP53* encoded by exon 4 to 8, which is important for the transcription-dependent und transcription-independent activity of p53 protein: 42% of patients carrying a mutation in this region die within one year as opposed to

18% of those with other mutations (136,137).

As mutant p53 variants have a longer half-life than the wild type, apparent overexpression—or rather: increased detection—of p53 protein is due to *TP53* mutations and thus to functionally altered p53 protein in most cases (138,139), which may interfere with physiological overexpression and hamper its effect in response to DNA damage and other stressors. In addition to its own direct effects, mutated p53 enhances the effect of Myc rearrangements (138).

To maintain its normal equilibrium, p53 is continuously bound by Mdm2, transported to the cytosol and ubiquitinated for degradation in the proteasome. The proteasome activator PA28γ enhances this process, further reducing the p53 level (140). PA28γ serum levels can be detected by a sandwich ELISA (141), and overexpression of PA28γ was found in various tumours such as colon (142), breast (143), liver (36) and thyroid cancers (144), which makes it likely to be involved in a mechanism of carcinogenesis.

Its role appears to be more complex, though: Live cell imaging has shown that 20S proteasomes locate on the site of DSB, which depends on ATM and PA28γ (145). In this process, the proteasome activator is bound and depleted by ATM (146), and this in turn enhances the accumulation of DNA repair proteins such as MDC1, BRCA1 and 53BP1. Interestingly, in this process γH2AX was only found in later stages after silencing of PA28γ. Thus it is required for the temporal DSB induction (145).

Through its signal in various cancers and its role in DNA repair, PA28γ has emerged as a candidate prognostic biomarker for neoplastic diseases. However, it has not yet been reported on in the context of lymphoma.

Conclusions

The genetic heterogeneity of malignant disease, including aggressive B-Non-Hodgkin Lymphomas such as DLBCL, leads to highly variable results of cytotoxic therapies, ranging from outright resistance to cure. The term "personalized medicine" is mostly used in the context of molecularly targeted substances requiring for their action the presence of a specific altered gene product. However, there is a number of potential biomarkers for monitoring DNA damage and thereby possibly predicting the efficacy of "classical" cytotoxic therapy. In the future this may lead to personalized application of conventional chemotherapy, too, which still is a mainstay of therapy in leukaemia,

lymphomas and cancers.

A selection of potential biomarkers has been compiled in this review to shed light on their biological role and potential use as a marker, always with a view on the analytical systems necessary for their detection and quantification.

High levels of the DNA DSB marker γH2AX were found in different tumour types. This biomarker is suitable for the early detection of cancer development because patients with oncological diseases have higher γH2AX foci than healthy volunteers (7,33,48-50,55) and γH2AX has already been investigated in the context of DLBCL (68). From theoretical and practical points of view, it appears to be an attractive candidate biomarker, not least because there are established methods available for the computerized analysis of γH2AX foci in PBMC (51,57,73,75,164,166-170,191). Nevertheless, γH2AX does not provide fully accurate information about the repair of this DNA damage. For this reason, further biomarkers are needed for a study to obtain an exact overview of the course of cancer and DLBCL in particular.

The broad substrate range of ATM (33,48,83) is a relevant example for the close connection of cell-cycle regulation, DNA repair, and apoptosis with cancer. Mutations in the ATM gene also promote DLBCL and complete loss leads to genomic instability and increased sensitivity to radiation (85-87). Like ATM, ATR phosphorylates the histone variant 2AX and is associated with increased radiation sensitivity. Both can be detected by immunostaining (103) and operate in the same signalling pathway (65,131,132,149). They can contribute to the development of tumours in case of alterations. In addition, DNA-PK is also involved in the activation of H2AX and maintains a low level of γH2AX, even if the kinase ATR is knocked out (78). ATR also activates p53 and phosphorylates BRCA1 (147,148). The three kinases are thereby involved in the recognition and repair of DNA damage within a complex network.

BRCA1 is associated with genotoxic stress and γ-radiation and increased γH2AX values could be detected in BRCA1 mutant breast cancer cells (69,95,96). The biomarker is primarily associated with breast and ovarian cancer, but by being associated with γH2AX (69), Rad51 and 53BP1 (65,66), involvement in DLBCL is also likely.

The DNA damage control point protein 53BP1 is involved in DNA repair and copy loss in the 53BP1 locus counts as a predisposing event in DLBCL (157). A correlation of 53BP1 with γH2AX and BRCA1 could be

found in breast cancer (69). It appears likely that all three also exert an influence on DLBCL. Complex formation of 53BP1 with Rad50 and Rad51 could also be found. The latter is involved in HRe and also a target for p53 and BRCA1 (124,126). It mediates DNA repair, cell cycle progression and replication and is particularly strongly induced by radiation. In addition, a positive correlation with γH2AX is known in endometrial cancer (129,165). A relationship between Rad51 and B-cell lymphoma could only be found in DT40 chicken cells (134). However, an association is likely and as a marker for HRe, Rad51 would complete an overview of DNA repair. Since oxidative stress can have a major impact on cancer development, 8-OHdG is also of relevance and strongly associated with cancer and DNA damage (110,111). Increased levels could be observed in lymphoma, prostate and bladder cancer (117,118) and a lower 8-OHdG level was associated with longer progression-free survival in DLBCL patients (120).

Overcoming the apoptotic signalling pathway is an important step in promoting cancer progression. Most cancers have elevated levels of Myc, which can lead to overexpression of BCL-2 and thus promote anti-apoptosis in cancer. These two are therefore key players in tumour development and are of predictive value in DLBCL, in particular when both are affected ("double-hit") (104,185,186). Another apoptosis suppressor and regulator of proliferation is STAT3, which is also involved in the regulation of Myc (97). STAT3 is considered a strong oncogene when misdirected and supports apoptosis resistance in ABC subtypes of DLBCL (105,106). Here the prognosis seems to depend on the phosphorylation pattern, since pSTAT3 carries a poor 5-year OS as opposed to pYSTAT3 which goes along with better survival. A mutation in the p53 gene *TP53* is present in 21% of DLBCL patients (135-137), which alters the function of p53 and can therefore also enhance the re-arrangement of Myc (138). p53 is degraded by proteases, including PA28γ (140). Overexpression of this protein has been detected in various tumours (36,142-144). However, it seems to have a far more complex role than has been observed so far, since 20S also localise proteasomes at DSB, a mechanism that depends on PA28γ and ATR and enhances BRCA1 and 53BP1 (145,146).

Most of the biomarkers described here can already be detected by IIF, FISH or IHC and are summarized in *Table 1*. A combination or multiplex method to check the course of the disease and its development in DLBCL patients would be possible. We were able to show that many biomarkers exert mutual influence on each other and thus a broad spectrum would be required to provide complete and rapid early detection of oncological diseases.

Footnote

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