Alternative pre-mRNA splicing leads to potential biomarkers in diffuse large B-cell lymphoma – a systematic review

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ABSTRACT

INTRODUCTION: The study of potential biomarkers in haematological malignancies has gained momentum in past decades. We compiled a systematic review to outline potential biomarkers based on alternative pre-mRNA splicing that were suggested to be clinically useful for the diagnosis, prognosis and response to therapeutics in diffuse large B-cell lymphoma (DLBCL).

METHODS: A comprehensive search of the literature in PubMed, Embase and Scopus was performed and supplemented with screening of reference lists. Only articles concerning potential biomarkers originating from reports on alternative pre-mRNA splicing were included. The contributions of these studies will help develop personalised medicine. Therefore, the clinical utility of the potential biomarkers was evaluated.

RESULTS: A total of 16 studies were included of which eight described seven different potential diagnostic biomarkers. Eight studies reported two potential prognostic biomarkers for CD44, its spliced mRNA variants and the resulting proteins that were the most frequently reported. Furthermore, two studies reported two proteins originating from alternative pre-mRNA splicing as potentially predictive biomarkers.

CONCLUSIONS: Alternative pre-mRNA splicing is a promising potential diagnostic, prognostic and predictive biomarker for the identification of pathogenic impacts in DLBCL. The use of these potential biomarkers in the clinical management of DLBCL awaits prospective clinical validation supporting its potential to contribute to the shift towards more personalised medicine.

Diffuse large B-cell lymphoma (DLBCL) is a highly malignant haematological disease. Despite the introduction of the combination chemotherapy rituximab, cyclophosphamide, doxorubicine, vincristine and prednisone (R-CHOP), a large number of DLBCL patients have non-curable cancer associated with poor survival. Thus, research aiming to improve the outcome for these patients is necessary [1, 2]. The goal is to prescribe the correct medicine for each patient with the correct dose at the correct time, also known as personalised medicine. An approach towards this is the use of biomarkers. This approach could be based on alternative pre-mRNA splicing.

The classic flow of genetic information is from DNA through RNA via transcription, before the effector protein is generated by translation. The initial product of transcription is pre-mRNA that is modified to form many different transcripts in a process of selective inclusion or removal of exons. This mechanism is defined as alternative pre-mRNA splicing. Alternative pre-mRNA splicing ensures a high diversity of the resulting effector proteins because different protein isoforms with different functions can be generated from the same pre-mRNA [3, 4]. Misregulated alternative pre-mRNA splicing can, however, also contribute to malignant transformation, cancer progression and metastasis by activating oncogenes and inactivating tumour suppressors [5, 6]. In general, the characteristics of cancers, or "hallmarks of cancer", have key elements that are alternatively spliced [5]. The role of alternative pre-mRNA splicing in DLBCL remains, however, largely unexplained [7, 8]. Based on the known role of alternative pre-mRNA splicing in the "hallmarks of cancer", its use as a biomarker and target for a potential new class of anticancer therapeutics has been proposed [3, 5, 6].

Recognition of aberrations in splicing events and their associations with disease are widely acknowledged in a great number of human diseases [9], including neurological diseases [10], muscular dystrophy [11] and myelodysplastic syndrome [12]. Because deregulated alternative pre-mRNA splicing is known to occur in DLBCL, this has been proposed as a potential biomarker [13]. To our knowledge, the literature concerning alternative pre-mRNA splicing as a potential biomarker in DLBCL has, however, never been studied systematically.

A biomarker is defined by the National Institutes of Health (NIH) as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [14]. Biomarkers may be classified as diagnostic (identifying patients with an abnormal condition), prognostic (indicator for overall outcome) or predictive (therapeutic response prior to an

SYSTEMATIC REVIEW

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KEY POINTS

Alternative pre-mRNA splicing is a process of selective inclusion or removal of exons in the pre-mRNA to ensure high diversity of the effector proteins.

Alternative pre-mRNA is known to occur in diffuse, large B-cell lymphoma (DLBCL), but its specific role remains unexplained.

Sixteen studies have found alternative pre-mRNA splicing to be a diagnostic, prognostic or predictive biomarker in DLBCL, but the clinical use of alternative pre-mRNA splicing is still long-termed.
It is hypothesised that personalised treatment of DLBCL patients may be based on alternative pre-mRNA splicing in the future.

intervention) [14, 15]. To assess the potential clinical usefulness of diagnostic biomarkers, a systematic approach guiding the process of biomarker development was developed by the Early Detection Research Network (ERDN) [16]. Currently, nothing similar has been designed for prognostic or predictive biomarkers.

Some biomarkers are already used in haematological, clinical indices such as the International Prognostic Index (IPI) [17]. The more recent classifications of DLBCL into "activated B-cell-like" (ABC), "germinal-centre B-cell-like" (GCB) and an unclassified third type based on tumour gene expression profiling [18] are widely recognised, but not implemented in a routine clinical setting. Other promising novel biomarkers are miRNAs [19] and the B-cell associated gene signatures [20].

The ultimate goal when constructing a clinical test based on a biomarker, whether it is diagnostic, prognostic, or predictive, is that a high sensitivity and specificity for detecting and distinguishing between positive (diseased, poor prognosis, non-responder to treatment) and negative (non-diseased, good prognosis, responder to therapy) cases. Therefore, we find it relevant to evaluate biomarker studies in terms of their sensitivity and specificity to assess their potential contribution towards a shift into personalised medicine.

This systematic review aimed to evaluate alternative pre-mRNA splicing as clinically useful diagnostic, prognostic or predictive biomarkers in DLBCL by evaluating the strength and limitations of the study design, the evidence level, the potential sensitivity and specificity, and the potential contribution to personalised medicine.

METHODS

This review was organised according to the Preferred Reporting for Systematic Reviews and Meta-analyses (PRISMA) guidelines [21].

Search strategy

A systematic search for studies was performed in Embase, PubMed and Scopus. According to the Population-Intervention-Comparison-Outcome (PICO) approach [22], the search was structured by combining MeSH terms/EMTREE terms and/or free-text words related to the population (such as diffuse large B-cell lymphoma), intervention (such as RNA splicing) and outcome (such as biomarker) (Appendix A). No terms were searched for in the comparison category as it was not relevant for this review. The reference lists of all included studies were searched for additional studies that the electronic search strategy may have missed. The last literature search was performed on 30 June 2015.

Study selection

To identify relevant articles meriting full review, titles and abstracts retrieved by the electronic search were screened. Articles of interest that met the inclusion criteria (see below) were subsequently reviewed in full length before inclusion. Reports on alternative splicing as a potential diagnostic, prognostic or predictive biomarker in DLBCL were considered for inclusion if they had alternatively spliced pre-mRNAs or resulting protein isoforms as an endpoint. To narrow the review, it was predefined to report only alternative pre-mRNA splicing and not mutations resulting in deregulated splicing machinery leading to alternative pre-mRNA splicing. Moreover, papers reporting alternatively spliced pre-mRNAs or resulting proteins as a target for therapeutics were not included as pharmacodynamics were not the main focus in this review. Reports concerning protein isoforms resulting from post-translational modifications were also excluded. Only original, full journal publications were included. In addition, the selection was carried out without limitations regarding study design, publication year or language.

Data extraction

To extract relevant information, a predesigned data abstraction form was used based on the Strengthening the Reporting of Observational studies in Epidemiology: Molecular Epidemiology (STROBE-ME) guideline [23]. The methodological quality was assessed by evaluating the limitations and strengths of each study [23-25] because no validated tool currently exists.

RESULTS

Search results and selected publications

Through database and reference list searching, 165 articles were identified (**Figure 1**). The search results from each database were imported into the reference manager programme Mendeley, and 27 duplicates were automatically removed. Therefore, 138 articles were the starting point for the analysis. By reviewing titles and abstracts, 22 papers fulfilled the inclusions criteria and were eligible and relevant for this analysis. A total of six

studies were excluded from the systematic analysis because only their abstracts were available. Thus, 16 full text articles remained for analysis.

Study characteristics of included studies

The eight studies reporting potential diagnostic biomarkers [26-33] were observational and cross-sectional due to the expression level being reported at one time point [34] (**Table 1**). The data sources varied from two cell lines to 250 tissue samples, comparing normal cell lines or other neoplastic cell line samples.

Two studies reported a potential biomarker to be both prognostic and predictive [35, 36] (**Table 2**). In general, all the studies reporting potential prognostic or predictive biomarkers were observational identifiers for the level of biomarker expression associated with an outcome or response to treatment. A total of seven studies were characterised as longitudinal studies with retrospective data from 28 to 290 tissue samples, and/or cell lines and clinical data from databases or medical records [13, 35-40]. One study had a cross-sectional design that examined the prognostic value of the potential biomarker by comparing the expression level of the potential biomarkers with the number of patients who survived by the end of the study period [41].

To assess any contribution to the shift towards personalised medicine and the potential sensitivity and specificity, the statistical methods in these studies were evaluated. Only studies reporting potential prognostic or predictive biomarkers that described the statistical methods used were included (Table 1). In general, these studies used simple statistical methods, such as correlation coefficients. Confidence intervals were reported only in one of the studies [36]; however, the significance value was reported in all studies [13, 35-41]. None of the studies presented pre-study power calculations. The sensitivity and specificity of the tests were stated only in Nagel et al by receiver operating characteristics (ROC) curves [37].

Results from individual studies

Potential diagnostic biomarkers

Several potential diagnostic biomarkers were investigated in different ways and a few studies examined the same potential biomarker [32, 33]. Several studies identify the presence of a potential promising diagnostic biomarker by comparing the expression level to normal or other neoplastic cells [26-28]. Two studies explore the distribution of alternative pre-mRNA splicing events between ABC- and GCB-DLBCL [27, 32]. These potential biomarkers were therefore hypothesised to be related to a worsened or improved outcome for ABC- and GCB-DLBCL, respectively [18]. Additionally, two studies investigate the presence of alternative pre-mRNA splicing in two subtypes of DLBCL, namely primary mediastinal large B-cell lymphoma (PMLBCL) and primary central nervous system lymphoma (PCNSL) that generally have a very poor prognosis compared with systematic DLBCL [31, 33]. They report that alternative pre-mRNA splicing in particular is present in these lymphomas compared with DLBCL.

Potential prognostic biomarkers

When considering the expression of potential prognostic or predictive biomarkers in the identified studies, a predominance of studies reporting alternatively spliced variants of CD44 were observed. Several studies describe that the alternative splicing of CD44 was significantly correlated with clinically accepted prognostic staging methods such as Ann Arbor Stage and IPI in DLBCL [13, 35, 37-40].

The identified studies use different endpoints for evaluation of the prognostic potential of CD44 splice variants (Table 2). All of the studies [13, 35-41] describe a high expression of CD44 splice variants correlated with a poor prognosis, except for Wei et al, who report that CD44v6, a specific alternatively spliced variant of CD44, was associated with superior survival in a multiple Cox regression analysis [35]. Furthermore, two studies re-

FIGURE 1

Flow chart outlining the selection procedure used to identify the included 16 full-text articles in this systematic review. All of the five articles identified by review of reference lists were included in the final qualitative synthesis.

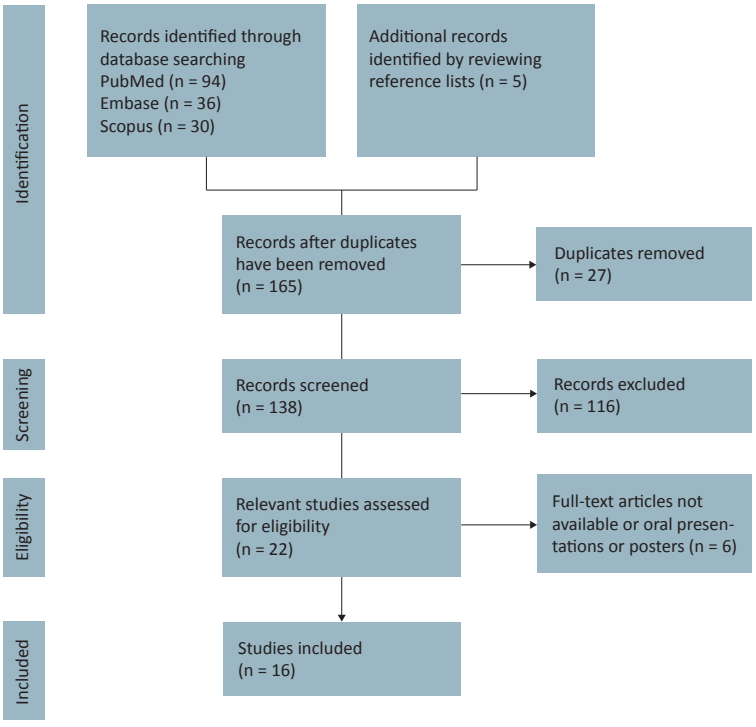


TABLE 1

The characteristics of each study reporting alternatively spliced potential diagnostic, prognostic, or predictive biomarkers are listed. The study design, data source, description of participants and control, study size, data measurement, subgrouping, and statistical methods were extracted from the full-text articles.

Reference	Study design	Data source	Participants and study size
Salles et al, 1993 [26]	Cross-sectional study	Tissue samples Cell lines	11 biopsies from primarily DLBCL patients in different disease stages at 2 hospitals Treatment status NR 7 cell lines of different origin as controls
Aguiar et al, 1999 [27]	Cross-sectional study	Tissue samples Cell lines	5 DLBCL cell lines 4 cell lines of different origin as controls
Greeve et al, 2003 [28]	Cross-sectional study	Tissue samples	1 biopsy from a primarily DLBCL patient at 1 hospital Normal and other neoplastic biopsies as controls Treatment status NR
Bende et al, 2002 [29]	Cross-sectional study	Tissue samples	1 biopsy from a primarily DLBCL patient at 1 hospital Other neoplastic biopsies as controls Treatment status NR
Liggins et al, 2004 [30]	Cross-sectional study	Tissue samples Serum samples Cell lines	6 paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital 10 serum samples from DLBCL patients 5 DLBCL cell lines Treatment status NR
Zamò et al, 2005 [31]	Cross-sectional study	Tissue samples	13 biopsies from PMLBCL patients in different disease stages at 1 hospital 150 biopsies from primarily DLBCL patients in different disease stages at 1 hospital Treatment status NR Normal and other neoplastic biopsies as controls
Brown et al, 2008 [32]	Cross-sectional study	Tissue samples Cell lines	52 biopsies from primarily DLBCL patients in different disease stages at 1 hospital 10 DLBCL cell lines Untreated
Courts et al, 2009 [33]	Cross-sectional study	Tissue samples Cell lines	19 frozen biopsies from primarily PCNSL patients in different disease stages at 1 hospital 24 paraffin-embedded biopsies from primarily PCNSL patients in different disease stages at 1 hospital Treatment status NR 2 cell lines (1 ABC-DLBCL and 1 GCB-DLBCL)
Drillenburg et al, 1999 [13]	Longi-tudinal study	Tissue samples from 1981-1989 Clinical data from database from a 15-year follow-up period	276 paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 15 hospitals Unspecific treatment
Inagaki et al, 1999 [40]	Longi-tudinal study	Tissue samples from 1986-1997 Clinical data from a 105-month follow-up period	42 paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital CHOP treatment
Tzankov et al, 2003 [38]	Longi-tudinal study	Tissue samples Clinical data from a 130-month follow-up period	82 formalin-fixed, paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital Untreated Controls: normal tonsils
Espinosa et al, 2006 [36]	Longi-tudinal study	Tissue samples from 1991-2002 Clinical data from database from a 160-month follow-up period	76 paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital CHOP or similar treated
Fridberg et al, 2007 [41]	Cross-sectional study	Tissue samples from 2001-2006 Cell lines Clinical data from a 6-year follow-up period	28 paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital Treatment status NR 5 DLBCL cell lines of GC origin 6 cell lines with various B-cell differentiation stages
Nagel et al, 2010 [37]	Longi-tudinal study	Tissue samples Clinical data from a 43-month follow-up period	290 formalin-fixed, paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital CHOP or similar treated, biopsy pretreatment
Min et al, 2011 [39]	Longi-tudinal study	Tissue samples from 1996-2003 Clinical data from medical records from a 60-month follow-up period	40 biopsies from primarily DLBCL in different disease stages at 1 hospital Treatment status NR
Wei et al, 2014 [35]	Longi-tudinal study	Tissue samples from 1998-2008 Clinical data from a 150-month follow-up period	117 formalin-fixed, paraffin-embedded biopsies from primarily DLBCL patients in different disease stages at 1 hospital CHOP/R-CHOP treated

ABC = activated B-cell-like; CD44s/H = cluster of differentiation 44 protein standard isoform; CD44v6 = cluster of differentiation 44 protein containing variant 6; CHOP = cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone containing therapy; DLBCL = diffuse large B-cell lymphoma; ELISA = enzyme-linked immune-sorbent assay; GC = germinal-centre; GCB = germinal-centre B-cell-like; NR = not reported; PCNSL = primary central nervous system lymphomas; PCR = polymerase chain reaction; PKC-β II = protein kinase C beta II; PMLBCL = primary mediastinal large B-cell lymphoma; PTP assay = protein tyrosine phosphatase assay; R-CHOP = rituximab-cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone containing therapy; RHAMM = hyaluronan-mediated motility receptor; ROC = receiver operating characteristics; RT-qPCR = reverse transcription quantitative polymerase chain reaction.

CONTINUES »

TABLE 1, CONTINUED

The characteristics of each study reporting alternatively spliced potential diagnostic, prognostic, or predictive biomarkers are listed. The study design, data source, description of participants and control, study size, data measurement, subgrouping, and statistical methods were extracted from the full-text articles.

Data measurement	Subgroups	Statistical methods
PCR	–	–
Flow cytometry RT-PCR Southern blot analysis	–	–
Immunomagnetic beads RT-PCR Ribonuclease protection assay	–	–
PCR	–	–
Multiple tissue expression arrays Multiple tissue Northern blots RT-PCR Immunohistochemistry	–	–
Immunohistochemistry Real time qPCR	–	–
Immunohistochemistry Western blotting array RT-PCR	–	–
RT-qPCR Immunohistochemistry Western blot	–	–
Immunohistochemistry	CD44s -/+ CD44v6 -/+	Spearman's correlation Survival curves with Kaplan-Meier method and log-rank test Multivariate analysis with hazard ratio and Cox proportional hazard model Cox regression with forward stepwise selection
Immunohistochemistry	CD44v6 -/+	Fisher's exact test Survival curves with Kaplan-Meier method and log-rank test Multivariate analyses with Cox stepwise proportional hazards model
Tissue microarray with immunohistochemistry	GC/non-GC CD44v6 -/+	Spearman's correlation Fisher's exact test Survival curves with Kaplan-Meier method and log-rank test Multivariate analysis using a general linear model
Immunohistochemistry	PKC-β II -/+ Membrane PKC-β II +/cytoplasm and nuclei PKC-β II +	Chi-squared test and Fisher's exact test Survival curves with Kaplan-Meier method with log-rank test Multivariate analyses with Cox stepwise proportional hazards model
Immunohistochemistry Western blot PCR PTP assay	GC/non-GC Dead/survived	Pearson chi-squared test Mann-Whitney test
Tissue microarray with immunohistochemistry	RHAMM -/+ CD95 -/+ RHAMM/CD44v -/+	Spearman's correlation Fisher's exact test Mann-Whitney test ROC-curves Survival curves with Kaplan-Meier methods and log-rank test Cox regression analysis
Immunohistochemistry	GC/non-GC CD44s -/+ CD44v6 -/+	Chi-squared test and Fisher's exact test Survival curves with Kaplan-Meier methods and log-rank test Multivariate analysis with Cox regression hazard model
Immunohistochemistry	CHOP/R-CHOP CD44H -/+ CD44v6 -/+	Spearman's correlation Mann-Whitney U-test Kaplan-Meier method Univariable and multivariable analysis with Cox regression analysis

TABLE 2

The potential prognostic correlations for biomarkers in DLBCL and the value of potential predictive biomarkers between groups that expressed or did not express the potential biomarker.

Biomarker	Diagnosis	Drug	Outcome measurement	Outcome	p-value	HR (CI 95 %)	Reference
Prognostic							
CD44s protein	DLBCL	–	OS	Worse	< 0.05/NS	1.3 (0.48-3.4)	[13, 35, 39]
			DFS	Worse	< 0.05	–	[13]
			Relapse rate	Worse	< 0.05	–	[13]
			EFS	Worse	< 0.05/NS	–	[35]
	DLBCL (CD10, bcl-6 positive)	–	OS	Worse	< 0.05	–	[38]
			Relapse rate	Improved	< 0.05	–	[38]
			FFS	Worse	< 0.05	–	[38]
	DLBCL, stage I	–	OS	Worse	< 0.05/NS	–	[13, 39]
	DLBCL, stage II	–	OS	–	NS	–	[13, 39]
	DLBCL, stage III	–	OS	Worse	< 0.05	–	[39]
	DLBCL, stage IV	–	OS	–	NS	–	[13]
	Nodal DLBCL, stage I	–	OS	Worse	< 0.05	5.07 (1.12-22.90)	[13]
	Extranodal DLBCL, stage I	–	OS	–	NS	–	[13]
	DLBCL (CHOP-treated)	–	OS	Worse	< 0.05/NS	0.93 (0.25-3.4)	[35]
			EFS	Worse	< 0.05/NS	0.76 (0.22-2.6)	[35]
	DLBCL (R-CHOP-treated)	–	OS	–	NS	1.6 (0.3-8.7)	[35]
			EFS	–	NS	1.3 (0.38-4.4)	[35]
CD44v4 protein	DLBCL (RHAMM-positive)	–	DSS	Worse	< 0.05	–	[37]
CD44v5 protein	DLBCL (RHAMM-positive)	–	DSS	Worse	< 0.05	–	[37]
CD44v6 protein	DLBCL	–	OS	Worse	< 0.05/NS	2.4 (1.2-4.7)	[13, 35, 39]
			EFS	Worse/improved	< 0.05	2.1 (1.1-1.4)	[35]
	DLBCL (CD44s protein negative)	–	OS	Worse	< 0.05	–	[38]
	DLBCL (RHAMM-positive)	–	DSS	Worse	< 0.05	–	[37]
	DLBCL (CHOP-treated)	–	OS	–	NS	2.1 (0.9-5.3)	[35]
			EFS	–	NS	2.1 (0.87-5.2)	[35]
	DLBCL (R-CHOP-treated)	–	OS	–	NS	2.3(0.6-8.5)	[35]
			EFS	–	NS	2.0 (0.69-5.9)	[35]
CD44v9 protein	DLBCL (RHAMM-positive)	–	DSS	Worse	< 0.05	–	[37]
PKC-β II protein	DLBCL	–	OS	–	NS	–	[36]
			DFS	Worse	< 0.05	1.9 (1.0-3.7)	[36]
			Poor survival	Worse	< 0.05	–	[40]
	DLBCL, stage I	–	DFS	Worse	< 0.05	3.7 (1.4-9.9)	[36]
PKC-β II protein (membranous)	DLBCL	–	OS	Worse	< 0.05	–	[36]
			DFS	Worse	< 0.05	–	[36]
Predictive							
PKC-β II protein	–	Adriamycin-containing chemotherapy	CR	Worse	< 0.05	–	[36]
CD44H protein	–	CHOP	OS	Worse	< 0.05	–	[35]
			EFS	Worse	< 0.05	–	[35]
	–	R-CHOP	OS	Worse	NS	–	[35]
			EFS	Worse	NS	–	[35]

bcl-6 = B-cell lymphoma 6 protein; CD10 = cluster of differentiation 10 protein; CD44s/H = cluster of differentiation 44 protein standard isoform; CD44v4/5/6/9 = cluster of differentiation 44 protein containing variant 4/5/6/9; CHOP = cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone containing therapy; CI = confidence interval; CR = complete remission; DFS = disease-free survival; DLBCL: = diffuse large B-cell lymphoma; DSS = disease specific survival; EFS = event-free survival; FFS = failure-free survival; HR = hazard ratio; NS = non-significant; OS = overall survival; PKC-β II = protein kinase C beta II; R-CHOP = rituximab-cyclophosphamide-hydroxydaunorubicin-oncovin-prednisone containing therapy; RHAMM = hyaluronan-mediated motility receptor.

port conflicting evidence as to whether or not alternative pre-mRNA splicing of CD44 is associated with increased or decreased relapse rates [13, 38].

Potentially predictive biomarkers

Two studies [35, 36] report alternative pre-mRNA splicing as a drug-specific, potentially predictive biomarker (Table 2). Espinosa et al [36] concluded that the PKC-β II membrane protein predicts a decreased complete remission (CR) rate when DLBCL patients were treated with adriamycin-containing chemotherapy. Wei et al [35] report that positive expression of the CD44H protein pre-

dict reduced overall survival (OS) and event-free survival (EFS) when treated with CHOP, while the predictive value is no longer present when treated with R-CHOP.

DISCUSSION

Key results

Several different, potential diagnostic biomarkers based on alternative pre-mRNA splicing were reported in these studies. However, only a few were repeatedly reported, and some contradictions between the studies were observed. Based on the study design, it is difficult to determine which of the current potential diagnostic candidate biomarkers merits selection over the others for further investigation.

All the studies reporting potential diagnostic biomarkers are in the early phases of diagnostic biomarker development, as outlined by the EDRN [16]; and their clinical implementation will require more studies at a higher developmental phase. Therefore, no potential diagnostic biomarker is currently considered as a promising biomarker in clinical studies for short-term use. Further research is recommended to identify new promising alternatively spliced biomarkers and such identification should be followed by the EDRN stages to develop a usable clinical biomarker.

Considering the potential prognostic biomarkers, only a few proteins originating from alternative pre-mRNA splicing were reported. CD44 alternative splicing is of special interest because this was widely described. In all studies reviewed, CD44 mRNA or CD44 protein expression was associated with a reduced OS [13, 35, 37-40], except in the study by Wei et al [35], where contradictory results were presented in one CD44 isoform. The reason for this discrepancy remains unclear; however, the authors hypothesise that the use of different antibodies and difference in staining/scoring between the isoforms may explain the difference. Two studies present two different potentially predictive biomarkers that were associated with inferior outcomes when treated with CHOP, or where the predictive value no longer existed after being R-CHOP treatment [35, 36].

Like the studies investigating potential diagnostic biomarkers, the studies reporting potential prognostic and predictive biomarkers were designed in a manner that postpones the medical utility of the biomarkers. Based on the study design, methods and statistical methods described, it was emphasised that this scientific field remains in the early phases of biomarker development, and the clinical usages are long-term. CD44 isoforms were, however, found repeatedly in many studies with positive results, warranting its potential validation in cross-centre retrospective studies or their inclusion in upcoming prospective trials.

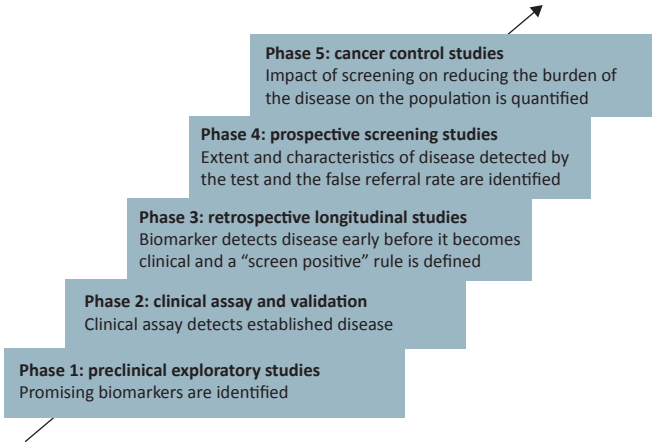
Critical assessment of the studies

The methodological quality of the selected studies was assessed according to the STROBE-ME guidelines which underline the importance of study transparency to achieve the best possible quality [23]. Several shortcomings in the included studies of this systematic review can be highlighted. First, inclusion and exclusion criteria are rarely stated in the articles [35, 36]. Explanations for in- and exclusion criteria are essential for assessing the internal validity of articles investigating prognosis because without such explanations, the influence of confounders and sensitivity or the specificity cannot be determined [25]. Another limitation was the incomplete reporting of loss to follow-up, which is only reported in three studies [13, 37, 38]. Without accurate reporting of loss to follow-up there is a risk of introducing bias to the observational study and therefore of overestimating or underestimating the effect. Various techniques were used in these studies to measure the expression level on the gene or protein levels. This contributed to the incomparability between the studies because no meta-analysis was performed. All of the studies reporting potential biomarker protein expression with immunohistochemistry used varying levels for reporting positive results, and the justification for the threshold level was reasoned only in some of these studies [13, 36, 37, 39-41]. The studies evaluating alternative splicing at the protein level instead of at the RNA level may be missing important, biologically relevant alternative splicing events because not all cases merely affect protein levels; the events may also alter protein function or localisation. In addition, changes in protein levels may be caused by other regulatory events in the cells; e.g. post-translational modifications. Therefore, studies directly measuring messenger RNAs are preferable.

Several studies failed to detail which statistical techniques were used, especially with respect to potential diagnostic biomarkers. This lack of detail is a methodological quality problem which along with the heterogenic reporting of results excluded the studies from meta-analysis. None of the studies reported pre-study power calculations. Some had small study sizes, and it with small study sizes it becomes increasingly likely that potentially significant associations remain undetected; thus, multiple analyses are less reliable. Only in the study by Nagel et al [37] stated that the sensitivity and the specificity of the diagnostic test had been tested. The most statistically valid method for combining test results, also used in the study by Nagel et al [37], is ROC curves [24]; and future diagnostic studies are recommended to report their results on sensitivity or specificity. The methodological problems outlined above warrants the conclusion that there is insufficient evidence for direct clinical use of the findings reported in the papers included in the present

FIGURE 2

Phases of prognostic or predictive biomarker development.



review. Due to these shortages, an assessment of the sensitivity and specificity was not possible.

Future research and recommendations

The introduction of alternatively spliced variants of mRNAs and/or the resulting proteins as biomarkers in the clinic would seem to demand that future research be conducted in predesigned stages of biomarker development like the one developed by the ERDN for diagnostic biomarker development [16]. Although similar phases have not been developed for prognostic and predictive biomarker development, the use of broad development phases is generally accepted. First, ideas for a future biomarker are conceived from, e.g., other cancers or genome-wide screening studies. Associations between biomarker expression and outcomes for prognosis or prediction are screened in retrospective material. Hereafter, construction of tests with prediction of dichotomised outcomes and cross-validation in the same retrospective material are performed. An independent retrospective dataset is used to validate the results before seeking clinical validation in a prospective, randomised clinical trial. Finally, a clinically validated biomarker may inform the decisions in personalised medicine (**Figure 2**) [42].

The emergence of new technologies gives rise to high expectations for future research within the field of alternative pre-mRNA splicing isoforms as potential biomarkers. One may consider whether a suitable diagnostic biomarker exists or whether it is at all possible to identify a single, alternatively spliced diagnostic biomarker given the complexity of most diseases. Several excluded, quite recent abstracts in this review examined the differentially expressed exons and splice variants in DLBCL with genome-wide exon arrays [7, 43, 44]. These studies are of great interest and much awaited because they may change the pace of research within alternatively spliced variants as biomarkers in DLBCL.

Critical assessment of the present review

The broadness of the search strategy and the comprehensiveness of the reported data are among the strengths of this systematic review. Furthermore, the review provides useful summaries of current knowledge and suggests additional recommendations for future research. As all studies are included regardless of their study design, study size or other methodological issues, this systematic review may be considered exhaustive regarding alternatively spliced mRNAs or the resulting proteins as potential biomarkers in DLBCL. The selected studies were all methodologically evaluated according to the STROBE-ME guideline [23], and the structure of this systematic review was based on the PRISMA guideline [21], which we believe further strengthens the reliability and quality of this review.

CONCLUSIONS

This systematic literature review collected all studies on alternative pre-mRNA splicing and the resulting protein isoforms as a novel biomarker candidate in DLBCL, evaluated the quality of the published studies and assessed their potential clinical use. Despite methodological heterogeneity among the included studies, our review identified a number of promising, alternatively spliced biomarkers for the study of pathogenic impact. In particular, CD44 isoforms are good candidates for further prospective testing. There is, however, insufficient evidence to recommend immediate use of any potential diagnostic, prognostic or predictive alternatively spliced biomarkers for DLBCL patients in the clinic. Even so, we believe that alternative pre-mRNA splicing will produce a potential biomarker in DLBCL following sufficient clinical validations; and we believe that this may play a key role in the shift towards an era characterised by personalised medicine, early diagnosis and accurate therapeutic choices.

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