Lectins: an effective tool for screening of potential cancer biomarkers

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In recent years, the use of lectins for screening of potential biomarkers has gained increased importance in cancer research, given the development in glycobiology that highlights altered structural changes of glycans in cancer associated processes. Lectins, having the properties of recognizing specific carbohydrate moieties of glycoconjugates, have become an effective tool for detection of new cancer biomarkers in complex bodily fluids and tissues. The specificity of lectins provides an added advantage of selecting peptides that are differently glycosylated and aberrantly expressed in cancer patients, many of which are not possibly detected using conventional methods because of their low abundance in bodily fluids. When coupled with mass spectrometry, research utilizing lectins, which are mainly from plants and fungi, has led to identification of numerous potential cancer biomarkers that may be used in the future. This article reviews lectin-based methods that are commonly adopted in cancer biomarker discovery research.

1 REVIEW ARTICLE

2 **Lectins: An Effective Tool for Screening of Potential Cancer**
3 **Biomarkers**

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15 **Abstract**

16 In recent years, the use of lectins for screening of potential biomarkers has gained increased

17 importance in cancer research, given the development in glycobiology that highlights altered

18 structural changes of glycans in cancer associated processes. Lectins, having the properties

19 of recognizing specific carbohydrate moieties of glycoconjugates, have become an effective

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21 specificity of lectins provides an added advantage of selecting peptides that are differently

22 glycosylated and aberrantly expressed in cancer patients, many of which are not possibly

23 detected using conventional methods because of their low abundance in bodily fluids. When

24 coupled with mass spectrometry, research utilizing lectins, which are mainly from plants and

25 fungi, has led to identification of numerous potential cancer biomarkers that may be used in

26   the future. This article reviews lectin-based methods that are commonly adopted in cancer

27   biomarker discovery research.

29   **Biology of lectins**

30   Lectins are carbohydrate binding proteins which are found ubiquitously in nature. The term

31   'lectin' originates from the Latin word *legere,* which means to choose or to select (*Boyd and*

32   *Shapleigh, 1954*). By binding to carbohydrates, lectins serve diverse biological functions.

33   Plant lectins, which typically cause agglutination of certain animal cells, play important roles

34   in defense against invasion of virus, bacteria or fungi (*Dias et al., 2015*). They are also

35   believed to mediate symbiosis relationship between plants and microorganisms (*De Hoff et*

36   *al., 2009*), and some may be involved in regulatory and signaling pathways in plant cells

37   (*Chen et al., 2002*).

38   Lectins have initially been classified based on their binding to different glycan structures.

39   They were categorized either as galactose, *N*-acetylglucosamine (GlcNAc), *N*-

40   acetylgalactosamine (GalNAc), glucose, L-fucose, mannose, maltose, sialic acid-specific or

41   complex glycan-binding lectins (*Lis and Sharon, 1986*). Later, they were also classified

42   based on the characteristics and numbers of their carbohydrate binding domains, namely

43   merolectins, hololectins, chimerolectins and superlectins (*Peumans et al., 2001*). With the

44   emergence of detailed structural properties of lectins being elucidated via the advancement of

45   technology, this classification further evolved into that based on distinct protein folding,

46   domains/structural similarities and evolutionary-relatedness of proteins (*Peumans et al.,*

47   *2001*). Via this categorization, 12 different lectin families, which include *Agaricus bisporus*

48   agglutinin homologues, amaranthins, class V chitinase homologues with lectin activity,

49   cyanovirin family, *Euonymus europaeus* agglutinin family, *Galanthus nivalis* agglutinin

50   family, jacalins, lysin motif domain, nictaba family, proteins with hevein domains, proteins

51   with legume lectin domains and ricin-B family (*Van Damme et al., 2008*), have been derived.

52   Ricin is believed to be the first lectin discovered in the seeds of the castor bean plant,

53   *Ricinus communis*, in 1888 (*Sharon and Lis, 2004*). Paradoxically, research on lectin only

54   flourished several decades subsequent to ricin's discovery after James Sumner successfully

55   purified a crystalline protein from jack bean (*Canavalia ensiformis*) in 1919. Sumner later

56   showed that the protein caused agglutination of cells such as erythrocytes and yeast. The

57   agglutinin, which is now known as concanavalin A or ConA, was also used for the first time

58   to demonstrate binding of lectins to carbohydrate. To date, there are more than a thousand

59   plant species that have been reported to possess lectins. Most of these lectins are in

60   abundance in seeds (*Lis and Sharon, 1986*; *Benedito et al., 2008*), whilst some are found in

61   leaves, roots, flower, sap, barks, rhizomes, bulbs, tubers and stems (*Dias et al., 2015*).

62   Because of their carbohydrate binding specificities, many lectins have been increasingly

63   applied in different areas of medical research and therapy (Table 1).

64

65   **Table 1** Summary of different applications of lectins in medical research and therapy

Lectin applications	Reference
Antibacterial agent	*Saha et al., 2014; Dias Rde et al., 2015*
Antifungal agent	*Klafke et al., 2013; Regente et al., 2014*
Antiparasitic agent	*Kobata-Kudo et al., 2005; Heim et al., 2015*

Antiviral agent	*Lusvarghi and Bewley, 2016; Monteiro and Lepenies, 2017*
Biomarker for disease detection and monitoring	*This review article*
Drug delivery	*Leong et al., 2011; Neutsch et al., 2013*
Induction of immunological and inflammatory response	*Singh et al., 2011; Ditamo et al., 2016*
Inhibition of cancer cell adhesion	*Redondo and Alvarez-Pellitero, 2010; Siva et al., 2014*
Inhibition of cancer cell growth / antitumor agent	*Jebali et al., 2014; Quiroga et al., 2015; Dan et al., 2016*
Promotion of healing in cutaneous wounds	*Brustein et al., 2012; Coriolano et al., 2014*

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Cancer biomarker

A biomarker is defined 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" (*Biomarkers Definition Working Group, 2001*). Hence, simple

parameters from pulse and blood pressure to protein constituents of cells, tissues, blood and

other biofluids are classified as biomarkers. Bodily fluids that have been mined for cancer

biomarkers thus far include serum/plasma, urine, saliva and other tissue-specific fluids such

as seminal fluid, cerebrospinal fluid, bone marrow aspirates, etc. Cancer biomarkers are

useful for early detection, diagnosis and prognosis of the disease. They are also heavily

relied on in management of patients, and assessment of pharmacodynamics of drugs, risk, as

well as recurrence of the disease.

79   Efforts in the search for new cancer biomarkers remain active even in the present day.

80   Currently, there are only a handful of cancer biomarkers that are commonly being used in the

81   clinical setting (Table 2), most of which have been officially approved by the US Food and

82   Drug Administration (FDA) for clinical use (*Füzéry et al., 2013*). More are definitely needed

83   for improved detection and diagnosis, particularly when the reliability of many of the FDA

84   approved biomarkers remains a problem due to their limited levels of sensitivity and

85   specificity. For example, CA-125 which is used as a biomarker for ovarian cancer, is also

86   often elevated in other cancers such as those of the breast (*Norum et al., 2001*), lung (*Salgia*

87   *et al., 2001*) and colon or rectum (*Thomas et al., 2015*). Similarly, prostate specific antigen

88   (PSA), a tissue-specific serum protein that is used in the diagnosis of prostate cancer, is also

89   commonly increased in sera of patients with benign prostatic hyperplasia, thus, posing

90   difficulties in clinically differentiating the two different conditions (*Barry, 2001*; *Thompson*

91   *et al., 2004*). These limitations, together with the recent development of various state-of-the-

92   art methodologies including genomics, proteomics and bioinformatics, have consequentially

93   propelled research towards identification of new cancer biomarkers that are more sensitive

94   and specific.

95

96   **Table 2** List of commonly used tumor markers in clinical practice.

Biomarker	Glycosylated	Cancer type	Specimen	Clinical use
Alpha-feto protein (AFP)	Yes	Testicular	Serum/plasma; Amniotic fluid[1]	Management of cancer
AFP-L3%	Yes	Hepatocellular	Serum	Risk assessment
Beta-2-microglobulin (B2M)	Yes	Blood cells	Serum, Urine, Cerebrospinal fluid	Monitoring progression and recurrence
Bladder tumor-	Unknown	Bladder	Urine	Monitoring disease

associated antigen				
CA 15-3	Yes	Breast	Serum/plasma	Monitoring disease; Response to therapy
CA 19-9	Yes[2]	Pancreatic	Serum/plasma	Monitoring disease
CA 27-29	Yes	Breast	Serum	Monitoring disease; Response to therapy
CA 125	Yes	Ovarian	Serum/plasma	Monitoring disease; Response to therapy
Carcinoembryonic antigen (CEA)	Yes	Colon	Serum/plasma	Monitoring disease; Response to therapy
c-Kit	Yes	Gastrointestinal stromal tumors	Tissue	Detection of tumor; Patient selection
EpCAM, CD45, cytokeratins 8, 18+, 19+	Yes	Breast	Whole blood	Monitoring progression and survival
Epidermal growth factor receptor (EGFR)	Yes	Colon	Tissue	Therapy selection
Estrogen receptor (ER)	Yes	Breast	Tissue	Prognosis; Response to therapy
HER2/NEU	Yes	Breast	Serum; Tissue	Monitoring progression; Therapy selection
Human chorionic gonadotropin	Yes	Testicular	Serum	Staging of cancer
Human epididymis protein 4 (HE4)	Yes	Ovarian	Serum	Monitoring progression and recurrence
Fecal occult blood (haemoglobin)	Yes	Colorectal	Feces	Detection of tumor
Fibrin/fibrinogen degradation product (DR-70)	Yes	Colorectal	Serum	Monitoring disease
Free prostate specific antigen	Yes	Prostate	Serum	Screening for disease
Nuclear mitotic apparatus protein (NuMA, NMP22)	Yes	Bladder	Urine	Diagnosis and monitoring disease
p63 protein	No	Prostate	Tissue	Differential diagnosis
Plasminogen activator inhibitor (PAI-1)	Yes	Breast	Tissue	Monitoring disease; Therapy selection

Progesterone receptor (PR)	Yes	Breast	Tissue	Therapy selection
Pro2PSA	Yes	Prostate	Serum	Discriminating cancer from benign disease
Thyroglobulin (Tg)	Yes	Thyroid	Serum/plasma	Monitoring disease
Total PSA	Yes	Prostate	Serum	Diagnosis and monitoring disease
Urokinase plasminogen activator (uPA)	Yes	Breast	Tissue	Monitoring disease; Therapy selection

97 [1]Also used in prenatal diagnosis of birth defects, a non-cancer application.

98 [2]A tetrasaccharide carbohydrate that is usually attached to *O*-glycans on the surface of cells.

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100

101   Amongst bodily fluids that have been mined for cancer biomarkers, serum/plasma is most

102   popular. Serum or plasma has the advantage of being routinely sampled in clinical

103   investigations. However, the extreme complexity and broad dynamic range of protein

104   abundance in serum and plasma pose a formidable challenge in research screening for

105   potential cancer biomarkers, which mostly comprise low abundance glycoproteins. Because

106   of this, many cancer biomarker exploratory studies involving serum or plasma often involved

107   enrichment and/or pre-fractionation of the samples using techniques such as

108   immunodepletion (*Preito et al., 2014*), immunoprecipitation (*Lin et al., 2013*) and size-

109   exclusion chromatography (*Hong et al., 2012*). However, the use of such techniques, despite

110   their wide applications in biomarker discovery investigations, is generally unable to make a

111   significant difference in unmasking proteins of low abundance [*Polaskova et al., 2010*], and

112   may result in concomitant loss of non-targeted proteins (*Bellei et al., 2011*).

113

114 **Applications of lectins in cancer biomarker discovery research**

115 Interestingly, the majority of cancer biomarkers that are currently being used in the clinical

116 settings are glycoproteins, which are structurally altered in their glycan moieties and

117 aberrantly expressed (*Henry and Hayes, 2012*). However, only alpha-fetoprotein (AFP) and

118 CA15-3 are clinically monitored for their glycan changes in the therapy for hepatocellular

119 carcinoma and breast cancer, respectively. The other cancer biomarkers are being monitored

120 for their total protein levels (*Kuzmanov et al., 2013*). Indeed, changes in glycosylation are

121 believed to be a main feature in oncogenic transformation as glycans are known to be

122 continuously involved in cancer evolving processes, such as cell signaling, angiogenesis,

123 cell-matrix interactions, immune modulation, tumor cell dissociation and metastasis.

124 Glycosylation changes that are commonly associated with cancer transformation include

125 sialylation, fucosylation, increased GlcNAc-branching of *N*-glycans, and overexpression of

126 truncated mucin-type *O*-glycans (*Pinho and Reis, 2015*). Hence, it is not surprising that

127 lectin-based approaches are becoming more popular in studies screening for novel cancer

128 biomarkers. Table 3 shows a list of lectins that have been used in cancer biomarker

129 discovery research. In the following sections of this review, the applications of lectins in

130 cancer biomarker discovery, including immobilized lectin affinity chromatography, enzyme-

131 linked lectin assay, lectin histochemistry, lectin blotting and lectin array, are addressed. For

132 lectin-based biosensor analysis, readers are recommended to refer to separate review articles

133 (*Pihíková et al., 2015*; *Coelho et al., 2017*).

134

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136

137   **Table 3** **List of lectins used in cancer biomarker discovery research.**

Lectin	Abbreviation	Specificity	Glycan Linkage	References
African legume (*Griffonia (Bandeiraea) simplicifolia*) lectin-I	GSLI (BSLI)	α-Gal; α-GalNAc	*O*-linked	*Lescar et al., 2002*
Asparagus pea (*Lotus tetragonolobus*) lectin	LTL	Fucα1-3(Galβ1-4)GlcNAc, Fucα1-2Galβ1-4GlcNAc	*N*-linked	*Pereira and Kabat, 1974; Yan et al., 1997*
Koji (*Aspergillus oryzae*) lectin	AOL	α1,6-fucosylated	*N*-linked	*Matsumura et al., 2007*
Castorbean (*Ricinus communis*) agglutinin	RCA	Galβ1-4GlcNAc; terminal β-D-Gal	*N*-linked	*Harley and Beevers 1986; Wang et al., 2011*
Champedak (*Artocarpus integer*) galactose binding lectin	CGB	Gal; GalNAc	*O*-linked	*Hashim et al., 1991; Gabrielsen et al., 2014*
Champedak (*Artocarpus integer*) mannose binding lectin	CMB	Man	*N*-linked	*Lim et al., 1997; Gabrielsen et al., 2014*
Daffodil (*Narcissus pseudonarcissus*) lectin	NPL	α-Man, prefers polymannose structures containing α-1,6 linkages	*N*-linked	*Kaku et al., 1990; Lopez et al., 2002*
Elderberry (*Sambucus nigra*) agglutinin	SNA	Neu5Acα2-6Gal(NAc)-R	*N*- and *O*-linked	*Shibuya et al., 1987; Silva et al., 2017*
Gorse or furze (*Ulex europaeus*) seed agglutinin-I	UEA-I	Fucα1-2Gal-R	*N*- and *O*-linked	*Holthofer et al., 1982; Raj Bharath and Krishnan, 2016*
Jackbean (*Canavalia ensiformis*) lectin	ConA	α-Man; α-Glc	*N*-linked	*Percin, et al., 2012*
Jackfruit (*Artocarpus heterophyllus*) lectin	Jacalin	Gal; GalNAc	*O*-linked	*Kabir, 1995; Jagtap and Bapat, 2010*
Lentil (*Lens culinaris*) hemagglutinin	LcH	Man; Glc (Affinity enhanced with α-Fuc attached to *N*-acetylchitobiose)	*N*-linked	*Howard et al., 1971; Chan et al., 2015*
Amur maackia (*Maackia amurensis*)	MAL II	Siaα2-3Galβ1-4GlcNAc; Siaα2-	*N*- and *O*-linked	*Konami et al., 1994;*

lectin II		3Galβ1-3GalNAc		*Geisler and Jarvis, 2011*
Orange peel fungus (*Aleuria aurantia*) lectin	AAL	Fucα1-6GlcNAc; Fucα1-3LacNAc	*N*- and *O*-linked	*Hassan et al., 2015*
Peanut (*Arachis hypogaea*) agglutinin	PNA	Galβ1-3GalNAc; Gal	*O*-linked	*Chacko and Appukuttan, 2001*; *Vijayan, 2007*
Chinese green dragon (*Pinellia pedatisecta*) agglutinin	PPA	Man	*N*-linked	*Li et al., 2014*
Poke weed (*Phytolacca americana*) mitogen lectin	PWM	GlcNAc oligomers	*N*-linked	*Kino et al., 1995*; *Ahmad et al., 2009*
Red kidney bean (*Phaseolus vulgaris*) lectin	PHA-L	Bisecting GlcNAc	*N*-linked	*Kaneda et al., 2002*; *Movafagh et al., 2013*
Thorn-apple (*Datura stramonium*) lectin	DSL	(GlcNAcβ4)n	*N*-linked	*Yamashita et al., 1987*; *Abbot et al., 2010*
Wheat germ (*Triticum vulgaris*) agglutinin	WGA	GlcNAcβ1-4GlcNAcβ1-4GlcNAc; Neu5Ac	*N*-linked	*Nagata and Burger, 1972*; *Parasuraman et al., 2014*
White button mushroom (*Agaricus bisporus*) lectin	ABL	GalNAc; Galβ1,3GalNAc (T antigen); sialyl-Galβ	*O*-linked	*Nakamura-Tsuruta et al., 2006*; *Hassan et al., 2015*

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140 **Immobilized-lectin affinity chromatography**

141   Immobilized-lectin affinity chromatography is a method for separation of glycoproteins

142   based on a highly specific interaction between a lectin, which is immobilized onto a chosen

143   matrix, and its carbohydrate ligands (*Hage et al., 2012*). The technique, when complemented

144   with mass spectrometry analysis, provides a useful tool in research aiming to identify

145   potential cancer biomarkers (Figure 1). By comparing bodily fluid samples of control

146   subjects with those from patients with cancer, glycoproteins that are aberrantly expressed or

147   differently glycosylated from the resulting glycoprotein-enriched eluates can be easily

148   identified. Immobilized-lectin affinity chromatography is currently one of the most widely

149   employed techniques for enrichment of glycoproteins in cancer biomarker research.

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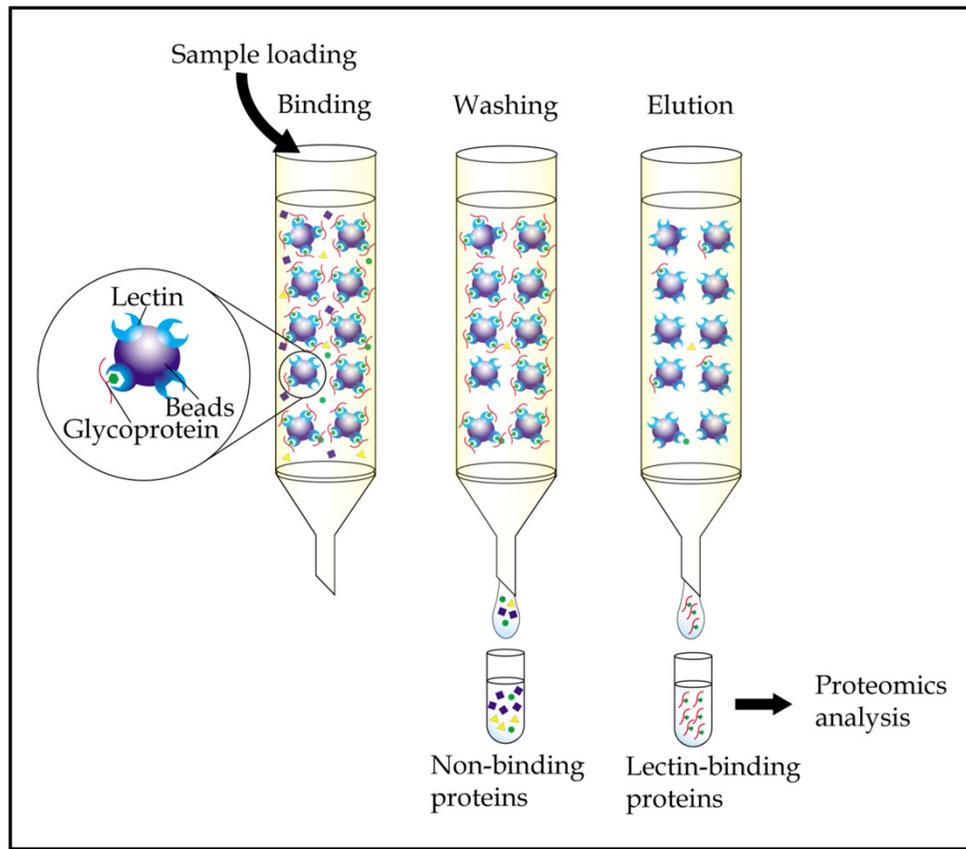
Figure 1 **General workflow of immobilized-lectin affinity chromatography.** Bodily fluid of cancer patients can be assayed for potential cancer biomarkers by running it through a chromatography column packed with a gel matrix that is conjugated with a lectin of interest. Non-binding proteins are then washed out, whilst bound glycoproteins are eluted using specific carbohydrate solutions. The lectin bound glycoproteins are finally identified using proteomics analysis.

By using immobilized-ConA, followed by separation by 2-dimensional gel electrophoresis (2-DE), *Rodriguez-Pineiro et al. (2004)* were able to profile serum samples of patients with colorectal cancer and showed significant altered expression of several *N*-glycosylated proteins that were identified by mass spectrometry. These included up-regulated expression of haptoglobin and lowered expression of antithrombin-III, clusterin,

164 inter-alpha-trypsin inhibitor heavy chain H4, beta-2-glycoprotein I and coagulation factor

165 XIII B chain in the colorectal cancer patients relative to healthy donors. Similarly,

166 *Seriramalu et al. (2010)* reported the lowered expression of complement factor B and alpha-2

167 macroglobulin in patients with nasopharyngeal carcinoma relative to controls using the

168 champedak mannose binding lectin. In the case of *O*-glycosylated proteins, considerable

169 studies have been reported using champedak galactose binding (CGB) lectin, which has a

170 unique characteristic of binding to the *O*-glycan structures of glycoproteins (*Abdul Rahman*

171 *et al., 2002*) in serum and urine samples. Cancers that have been investigated using

172 immobilized-CGB lectin include endometrial cancer (*Mohamed et al., 2008*) and prostate

173 cancer (*Jayapalan et al., 2012*). However, most of the serum and urine *N*- and *O*-

174 glycosylated proteins that were isolated using the immobilized-lectin affinity

175 chromatography are not directly cancer associated but the body's highly abundant acute-

176 phase reactant proteins (*Pang et al., 2010*).

177   More recently, analyses of enriched glycopeptide eluates of immobilized-lectin affinity

178 chromatography for identification of site-specific glycosylation using mass spectrometry

179 techniques have been reported in studies in search of potential cancer biomarkers.

180 Enrichment of core fucosylated peptides using *Lens culinaris* agglutinin (LCA) after trypsin

181 digestion of glycoproteins, followed by endo F3 partial deglycosylation and nano LC-

182 MS/MS methodologies, has led to identification of glycopeptides that can potentially be used

183 as diagnostic biomarkers for pancreatic cancer (*Tan et al., 2015*). Similarly, enrichment of

184 trypsin-digested glycopeptides using *Aleuria aurantia* lectin (AAL) that was immobilized

185 onto agarose gel, followed by analysis using LC/MS, has resulted in identification of alpha-

186 1-acid glycoprotein with multi-fucosylated tetraantennary glycans as a potential marker for

187 hepatocellular carcinoma (*Tanabe et al., 2016*). In another study, the *Sambucus niagra*

188 agglutinin (SNA) affinity column was used to separate various glycoforms of serum PSA

189 according to the types of sialic acid linkages (*Llop et al., 2016*). This has resulted in

190 identification of α2, 3-sialylated PSA as a marker for discriminating patients with high-risk

191 prostate cancer from those with benign prostatic hyperplasia and low-risk prostate cancer,

192 with higher levels of sensitivity and specificity.

193   Another variant of immobilized-lectin affinity chromatography used in cancer biomarker

194 research is multi-lectin affinity chromatography.   Since no single lectin is able to isolate the

195 complete complement of a glycoprotein, a multi-lectin affinity chromatography is gaining

196 popularity because of its greater coverage and depth of analyses. Using a combination of

197 four different types of lectins, including ConA, SNA, *Phaseolus vulgaris* agglutinin (PHA)

198 and *Ulex europaeus* agglutinin (UEA), for sequential multi-lectin affinity chromatography in

199 silica-based microcolumns and nano-LC/MS/MS for identification of proteins, *Madera et al.*

200 *(2007)* successfully profiled glycoproteins from microliter volumes of serum. Along the

201 same line but using ConA, wheat germ agglutinin (WGA) and jacalin that were integrated

202 into an automated HPLC platform and immuno-depleted serum samples, *Zeng et al. (2011)*

203 demonstrated a comprehensive detection and changes in the abundances of post-

204 translationally modified breast cancer-associated glycoproteins. To facilitate a cascading

205 flow of samples from column to column for simultaneous and efficient capturing and

206 enrichment of fucosylated proteins, *Selvaraju and El Rassi (2013)* developed of a platform,

207 which comprised multi-lectin columns driven by HPLC pumps for elucidating differential

208 expression of serum fucome between cancer-free and breast cancer subjects. This method

209 surpasses issues such as loss of samples due to sample preparation and processing (e.g.,

210   dilution) as well as other experimental biases that commonly occur when using other

211   techniques.

212   Recently, *Miyamoto et al. (2016)* reported a comprehensive proteomic profiling of ascites

213   fluid obtained from patients with metastatic ovarian cancer enriched by differential binding

214   to multiple lectins, including ConA, AAL and WGA. Alpha-1-antichymotrypsin, alpha-1-

215   antitrypsin, ceruloplasmin, fibulin, fibronectin, hemopexin, haptoglobin and lumican

216   appeared more abundant in ascites of the patients compared to controls. Further glycopeptide

217   analysis identified unusual *N*- and *O*-glycans in clusterin, fibulin and hemopexin

218   glycopeptides, which may be important in metastasis of ovarian cancer. Similar use of multi-

219   lectin affinity chromatography for enrichment of *N*-linked glycoproteins by *Qi et al. (2014)*

220   has successfully identified human liver haptoglobin, carboxylesterase 1 and procathepsin D

221   as candidate biomarkers associated with development and progression of hepatocellular

222   carcinoma. Whilst the concentrations of human liver haptoglobin and carboxylesterase 1

223   were consistently lower, higher concentration of procathepsin D was detected in the liver

224   cancer tissues. Further in-depth analysis projected the promising use of procathepsin D as

225   serological biomarker for diagnosis of hepatocellular carcinoma.

226

227   **Enzyme-linked lectin assay**

228   Enzyme-linked lectin assay is a method that adopts the principle of enzyme-linked

229   immunosorbent assay but uses lectin as one of the reagents instead of antibody. This method

230   was introduced by *McCoy Jr. et al. (1983)* in the early eighties. In a direct assay, samples

231   that contain glycoconjugates may be coated directly onto the wells of a microtiter plate,

232   followed by addition of an enzyme-conjugated lectin, which will then bind to their glycan

233   structures (Figure 2, panel A). The enzyme converts a colorless substrate solution to a

234   colored product, that is then measured using a spectrophotometer, and whose intensity is

235   used to estimate the levels of the coated glycoconjugates. Depending on the structures of

236   glycans that need to be detected, specific lectins are carefully selected. Enzyme-linked lectin

237   assay has been used in a plethora of research including those of cancer biomarkers

238   (*Kuzmanov et al., 2013*). It is easy to perform, very cost effective and requires minute

239   amounts of samples. One drawback of the direct enzyme-linked lectin assay is that

240   glycoproteins that are detected may not be identifiable unless it is coupled with proteomics

241   analysis or antibody detection.

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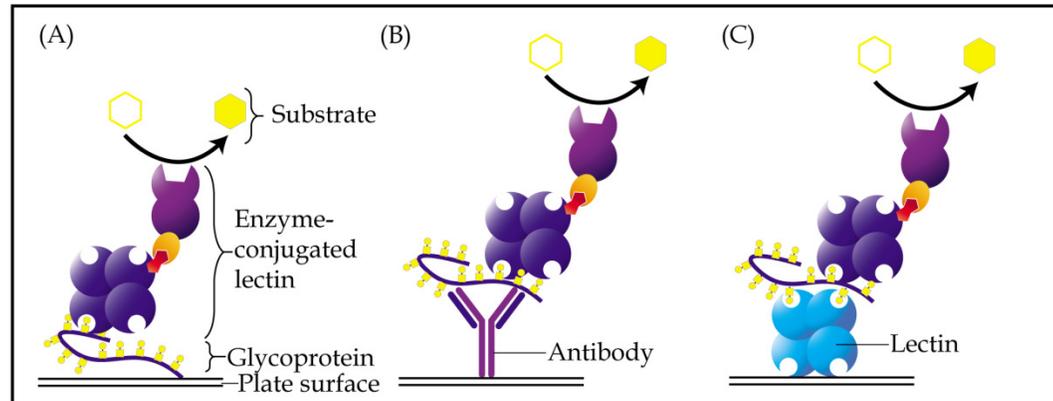
244   **Figure 2** **Different approaches of enzyme-linked lectin assay.** (A) In the direct assay,

245   coating of samples is performed directly onto the surface of a microtiter plate, followed by

246   addition of enzyme-conjugated lectin. (B) In the hybrid assay, antibody is instead coated onto

247   the plate to capture specific glycoproteins of interest, prior to addition of the enzyme-

248   conjugated lectin. (C) Sandwich enzyme-linked lectin assay is an alternative method involving

249   two different lectins. The first lectin is coated onto plates and used as a capturing reagent,

250   whilst the second lectin is used as detection reagent. For all the aforementioned methods,

251   glycoproteins are usually detected using a lectin that is conjugated to an enzyme, which then
252   converts a specific substrate into a colored product.

253

254   Based on their earlier study that identified a predominantly high molecular weight
255   glycoprotein that binds to peanut lectin (PNA) in the sera of patients with pancreatic cancer,
256   *Ching and Rhodes (1989)* developed a direct enzyme-linked PNA assay for diagnosis of
257   pancreatic cancer. Results obtained from the lectin-based assay were apparently found to be
258   comparable with those derived from using CA19-9 radioimmunoassay, in terms of sensitivity
259   and specificity for pancreatic cancer. In another study, *Reddi et al. (2000)* reported the use of
260   similar enzyme-linked PNA assay to estimate the levels of Thomsen-Friendenreich antigen
261   (T-Ag) in sera of patients with squamous cell carcinoma of the uterine cervix, before and
262   after radiotherapy. The study demonstrated significantly higher levels of T-Ag in the sera of
263   the uterine cervical cancer patients compared to normal individuals, and that the expression
264   of PNA-binding T-Ag were directly proportional to the aggressiveness of the cancer. In a
265   study by *Dwek et al., (2010)*, the specificity of UEA-1 lectin to α1,2-linked fucose sites was
266   capitalized for detection of fucosylated serum free PSA in a direct enzyme-linked lectin
267   assay. Their results demonstrated higher levels of fucosylated serum free PSA in patients
268   with prostate cancer compared to those with benign prostatic hyperplasia.

269   Aside from sera, direct enzyme-linked lectin assay has also been used in the analysis of
270   tissue lysate glycoproteins. In a recent study of breast cancer tissue lysates of different
271   stages, *Wi et al. (2016)* demonstrated increased interaction with ConA, *Ricinus communis*
272   Agglutinin I, AAL and *Maackia amurensis* lectin II (MAL II), relative to normal tissue
273   specimen of the same subjects. This is generally interpreted to show enhanced

274   mannosylation, galactosylation, sialylation and fucosylation of glycoproteins in the breast

275   cancer tissues. In another study, *Kim et al. (2014)* have shown lower levels of fucosylation

276   and sialylation of cytosolic intracellular glycoproteins in cancerous human cervical tissues

277   compared to normal tissue specimens from the same subjects using AAL and SNA lectins,

278   respectively. However, the levels of mannosylation, which was assayed using ConA, were

279   not significantly different between cancer tissues and normal specimens.

280   Subtle changes to the classical enzyme-linked lectin assay protocol have been introduced

281   over the years. An example is the combined use of antibody with lectin to enable detection

282   of glycosylation on a specific protein (*Kim et al., 2008*). In this case, an antibody may be

283   coated directly onto the wells of a microtiter plate, which will allow pre-capturing of a

284   protein of interest from complex samples (Figure 2, panel B). A lectin is then added and let

285   on to bind with the glycan structures of the protein. In this method, prior purification of a

286   glycoprotein is not needed as the antibody utilized specifically isolates the protein of interest

287   from within the samples. This method is also more suitable for glycoprotein antigens, which

288   are generally hydrophilic and cannot be well-coated onto a microtiter plate. The

289   disadvantage of this approach is that a lectin may directly interact with glycan chains of the

290   antibody used, which would then result in high background readings.

291   To solve the issue of the non-specific direct interaction of lectin to antibodies in enzyme-

292   linked lectin assays, *Takeda et al. (2012)* have instead used the Fab fragment of anti-human

293   haptoglobin IgG antibody and biotinylated AAL lectin for sandwich detection of fucosylated

294   haptoglobin. Their results showed that the levels fucosylated haptoglobin were significantly

295   associated with overall and relapse-free survival, distant metastasis, clinical stage, and

296   curability of patients with colorectal cancer. When Kaplan-Meier analysis was performed on

297   patients after more than 60 months of surgery, positive cases of fucosylated-haptoglobin

298   showed poor prognosis compared with fucosylated-haptoglobin negative cases. This leads to

299   the suggestion of fucosylated haptoglobin as a prognostic marker in addition to CEA for

300   colorectal cancer. Along the same line, *Jin et al. (2016)* have instead used protein A as the

301   capturing reagent and AAL lectin as detection probe, for assessment of fucosylated

302   circulating antibodies in cervical intraepithelial neoplasia and cervical cancer. Significantly

303   lower levels of fucosylated circulating immunoglobulins were shown in female patients with

304   cervical cancer compared to those with cervical intraepithelial neoplasia or normal subjects.

305   In a reverse contrast strategy, *Wu et al. (2013)* have used SNA lectin to capture sialylated

306   glycoproteins and biotinylated-antibodies to detect clusterin, complement factor H,

307   hemopexin and vitamin D-binding protein to validate the altered levels of the respective

308   glycoproteins in sera of patients with ovarian cancer. The results were consistent with their

309   data that was previously generated using isobaric chemical labeling quantitative strategy. In

310   a similar strategy, *Liang et al. (2015)* have used *Bandeiraea (Griffonia) simplicifolia*-I (BSI),

311   AAL and Poke weed mitogen (PWM) lectins as capturing reagents and biotinylated anti-

312   human α-1-antitrypsin polyclonal antibody in a sandwich enzyme-linked lectin combination

313   assay to validate results of their lectin microarray analysis of serum samples of patients with

314   lung cancer. While galactosylated α-1-antitrypsin was shown to demonstrate remarkable

315   discriminating capabilities to differentiate patients with non-small-cell lung cancer from

316   benign pulmonary diseases, their fucose- and poly-LacNAc-containing counterparts may be

317   used to discriminate lung adenocarcinoma from benign diseases or other lung cancer

318   subtypes, and small-cell lung cancer from benign diseases, respectively.

319 In a slightly different context, *Lee et al. (2013)* have developed a sandwich enzyme-

320 linked assay that uses two different lectins that both bind to *O*-glycan structures of

321 glycoproteins (Figure 2, panel C). The assay, which uses CGB lectin as capturing coated

322 reagent and enzyme-conjugated jacalin as detection probe, was primarily designed to

323 measure the levels of mucin-type *O*-glycosylated proteins in serum samples. When the assay

324 was applied on sera of patients with stage 0 and stage I breast cancer as well as those of

325 normal control women, significantly higher levels of *O*-glycosylated proteins were detected

326 in both groups of breast cancer patients (*Lee et al., 2016*). The specificity and sensitivity of

327 the assay were further improved when the same serum samples were subjected to perchloric

328 acid enrichment prior to the analysis. Further characterization of the perchloric acid isolates

329 by gel-based proteomics detected significant altered levels of plasma protease C1 inhibitor

330 and proteoglycan 4 in both stage 0 and stage I breast cancer patients compared to the

331 controls. Their data suggests that the ratio of the serum glycoproteins may be used for

332 screening of early breast cancer.

333

334 **Lectin histochemistry**

335 Like immunohistochemistry, lectin histochemistry is a microscopy-based technique for

336 visualization of cellular components of tissues except that it uses lectin instead of antibodies.

337 Utilization of labelled lectins in the tissue staining procedure limits the technique to detection

338 of only glycan-conjugated components, as well as those whose glycan moieties are being

339 recognized specifically by the individual lectins. Unlike immunohistochemistry which

340 detects presence of specific antigens based on the specificities of antibodies used, lectin

341 histochemistry provides information concerning glycosylation processes within a tissue

342   sample as well as their intracellular locations. This information can be very useful in the

343   characterization and/or detection of diseases.

344   In lectin histochemistry, labelling can be performed directly or indirectly (*Roth, 2011*).

345   In the direct labelled method, which is generally less sensitive than the direct method, lectins

346   are directly linked to fluorophores, enzymes, colloidal gold or ferritin, depending on the

347   microscopy involved (Figure 3, panel A). On the other hand, the indirect method involves

348   conjugation of lectins with biotin or digoxigenin, which may be detected using enzyme

349   linked-streptavidin or -anti-digoxigenin, respectively (Figure 3, panel B). Apparently, not all

350   chemicals can be used in the fixation and embedding of tissues in lectin histochemistry. For

351   example, the use of formaldehyde in fixation of tissue specimens is known to cause reduced

352   sensitivity of the *Griffonia simplicifolia* agglutinin, whilst ethanol-acetic acid fixation

353   improved its binding (*Kuhlmann and Peschke, 1984*). Paraffin, which causes denaturation of

354   proteins, is also known to result in attenuated binding of lectins due to sequestration of

355   carbohydrates in the glycoproteins that are denatured. However, this can be largely reversed

356   by removal of tissue-embedded paraffin using xylene or by trypsinization, which breaks the

357   protein cross-links and allows the lectins to bind more efficiently (*Brooks and Hall, 2012*).

358

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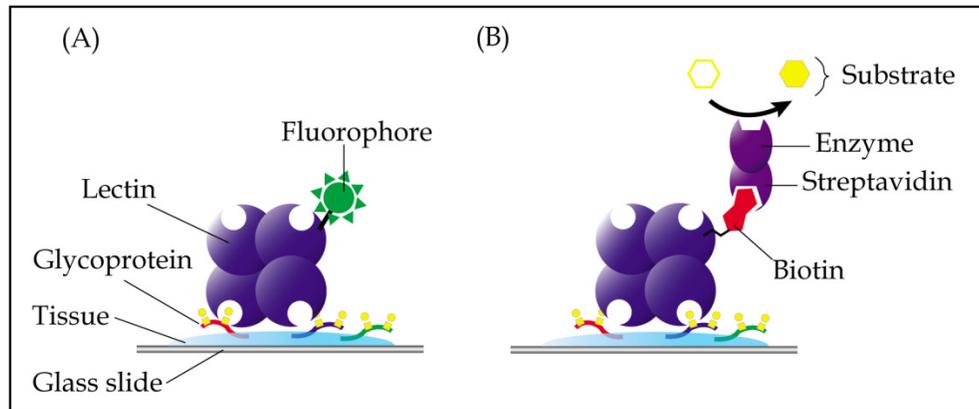
Figure 3 **Common techniques in lectin histochemistry.** Comparative staining of cancer
versus normal tissues may highlight aberrant glycosylation of glycoproteins. (A) In the direct
method, glycoproteins are detected in tissue specimens using a lectin that is covalently linked
to fluorophores, enzymes, colloidal gold or ferritin. (B) The indirect labelled method, which
is generally more sensitive, involves use of a lectin that is conjugated with a hapten, such as
biotin or digoxigenin, which are then recognized using enzyme linked-streptavidin or -anti-
digoxigenin, respectively.

Lectin histochemistry has been extensively used in the study of glycosylation changes in
cancer tissues. Two lectins have been found useful in distinguishing the different
histological grades of mucoepidermoid carcinoma, the most common type of salivary gland
cancer (*Sobral et al., 2010*). Whilst ConA was demonstrated to be able to stain all grades of
mucoepidermoid carcinoma tissues, staining with UEA-I lectin showed direct correlation of
malignancy with the intensity of staining. Another example is cholangiocarcinoma that is
attributed to the river fluke infection that commonly occurs in Thailand. In the study of the
parasite-induced cancer, *Indramanee et al. (2012)* have used multiple lectins to demonstrate
aberrant glycosylation of glycoconjugates in paraffin-embedded liver tissues of patients with
primary cholangiocarcinoma. Unique lectin staining patterns derived from the cancer

378   patients, relative to non-tumorous tissues, can be utilized as early stage markers for the bile

379   duct cancer. Similarly, SNA has been proposed for use as a prognostic probe for invasive

380   ductal carcinoma based on the different staining patterns that were generated compared to

381   tissue sections of patients with stage 0 breast cancer, ductal carcinoma in situ (*Dos-Santos et*

382   *al., 2014*). In another histochemical study, eight different lectins have been used to identify

383   specific carbohydrates that may contribute to the progression of colorectal cancer

384   (*Hagerbaumer et al., 2015*). The results showed changes in the binding patterns of five of

385   the lectins during advancement of metastasis from adenoma to colorectal carcinoma.

386

Lectin blotting

388   Lectin blotting is an extension of western blotting that uses lectin instead of antibody to

389   detect glycoconjugates (*Shan et al., 2001*). As in western blotting, samples are similarly

390   resolved using polyacrylamide gel electrophoresis and transferred onto a polyvinylidene

391   fluoride (PVDF) or nitrocellulose membrane but detected using glycan-specific lectin probes

392   (Figure 4). Like histochemistry, visualization of the lectin complex is enabled via the use of

393   conjugates such as enzymes, fluorescent dyes, biotin, digoxigenin, colloidal gold and

394   radioactive isotopes. In lectin blotting, the concentrations of lectins used must be at optimal

395   levels to reduce false-positive binding. Although a powerful tool, this technique is however

396   not quite suitable for routine diagnostics.

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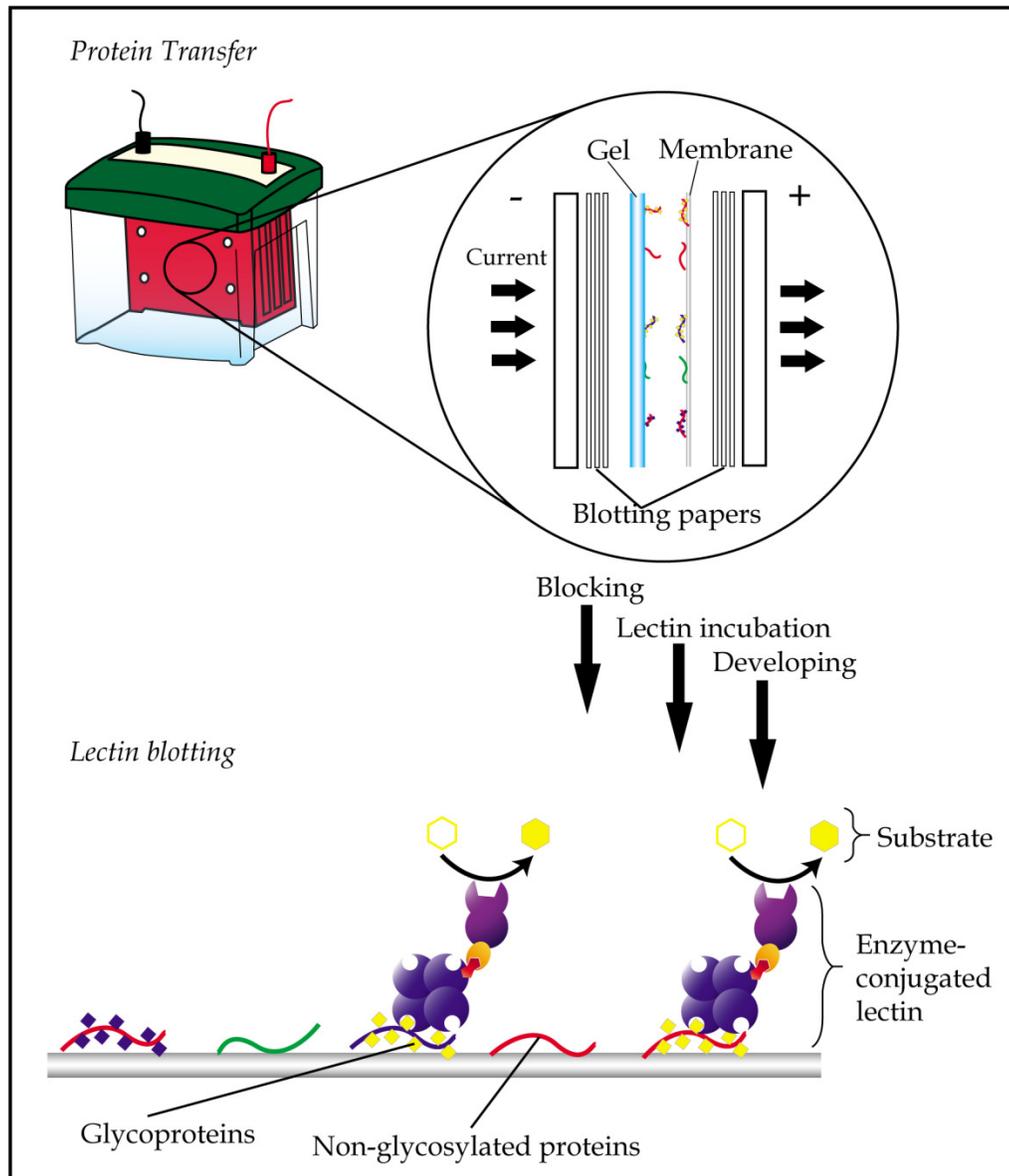
398

399   **Figure 4 General workflow of lectin blotting.** The method initially involves transferring of

400   proteins that are resolved by gel electrophoresis onto a PVDF or nitrocellulose membrane.

401   This is then followed by subjecting the membrane to washing, blocking and incubation with

402   lectins that are conjugated to an enzyme, a fluorescent dye, biotin, digoxigenin, colloidal gold

403   or radioactive isotopes. Comparative blotting of bodily fluids of cancer patients versus those

404   from cancer negative subjects may highlight presence of aberrantly glycosylated and/or

405   expressed glycoproteins.

406

407   In the past, lectin blotting studies have been especially useful in characterization of

408   structures of glycans (*Akama and Fukuda, 2006*), detection and quantification of *N*- and *O*-

409   glycosylated proteins (*Roth et al., 2012*) and detection of altered glycosylation following an

410   abnormality in glycosylation pathways due to disease processes (*Kitamura et al., 2003*). In

411   cancer biomarker studies, lectin blotting is often used for comprehensive profiling of

412   glycosylated proteins in biofluids. For example, the CGB lectin has been extensively used to

413   demonstrate altered abundances of various *O*-glycosylated proteins in serum and/or urine

414   samples of cancer patients that were resolved by 2-DE and transferred onto nitrocellulose

415   membrane. Cancers that have been investigated using the method include endometrial

416   cancer, cervical cancer (*Abdul-Rahman et al., 2007*), breast cancer, nasopharyngeal

417   carcinoma, bone cancer (*Mohamed et al., 2008*), ovarian cancer (*Mu et al., 2012*) and

418   prostate cancer (*Jayapalan et al., 2012*; *Jayapalan et al., 2013*). Similar lectin blotting

419   studies have also been applied on cell lines. Examples are the use of *Pinellia pedatisecta*

420   agglutinin-based lectin blotting analysis to generate unique glycosylation fingerprints for

421   leukemia and solid tumor cell lines (*Li et al., 2014*), and the utilization of ConA and CGB

422   lectin to demonstrate altered released of *N*- and *O*-glycosylated proteins from murine 4T1

423   mammary carcinoma cell line (*Phang et al., 2016*).

424   Another use of lectin blotting is as a means of validation of tumor-specific glycosylation.

425   Based on earlier results that showed elevated levels of mRNA of specific

426   glycosyltransferases in endometroid ovarian cancer tissue relative to normal ovary, *Abbott et*

427   *al. (2010)* have selected three different lectins (*Phaseolus vulgaris* erythroagglutinin, *Aleuria*

428   *aurantia* lectin and *Datura stramonium* lectin) with distinctive affinities for the respective

429   products of the enzymes to validate glycosylation changes of glycoproteins that are expressed

430   in the ovarian cancer tissues. By extracting intact glycoproteins from the ovarian tissues

431   before isolating the lectin-reactive proteins, the researchers were able to identify a total of 47

432   potential tumor-specific lectin-reactive markers. In another study, *Qiu et al. (2008),* using

433   biotinylated AAL and SNA lectin-blot detection method, were able to validate the

434   differential *N*-linked glycan patterns that are related to the levels of sialylation and

435   fucosylation of complement C3 in colorectal cancer patients, compared to those with

436   adenoma and normal subjects. Similarly, *Park et al., (2012)* have validated earlier findings

437   of aberration of fucose residues in haptoglobin β chain that is associated with progression of

438   colon cancer by generating comparable results using *Lotus tetragonolobus* and *Aspergillus*

439   *oryzae* lectins as detection probes in lectin blotting experiments.

440

441   **Lectin array**

442   Lectin array is a technique that was developed for rapid and sensitive analysis of glycans in a

443   high-throughput manner. The technique uses multiple lectins, which are mostly plant-

444   derived, that are immobilized onto a solid support at a high spatial density to detect different

445   carbohydrate content of glycoproteins or glycolipids in a single sample (*Hu and Wong, 2009*;

446   *Hirabayashi et al., 2011*). Display of the lectins in an array format enables observation of

447   the distinct binding interactions simultaneously, which then provides a unique method for

448   rapid characterization of carbohydrates on glycoconjugates (Figure 5, panel A). A glass slide

449   is the most common material used as solid support for the array application. Lectins are

450   coated on the glass surface either by covalent interaction or physical adsorption. Glass slides

451   are usually pre-treated with chemical derivatives such as *N*-hydroxy succinimidyl esters (*Hsu*

452   *and Mahal, 2006*), epoxides (*Kuno et al., 2005*), biotin, streptavidin (*Angeloni et al., 2005*),

453   and 3D hydrogels (*Charles et al., 2004*). Each droplet of lectin is printed onto the glass slide

454   and arranged according to a specific grid map using an array printer. The printed slide is

455   held in place by a multi-well gasket, which allows samples to be loaded into each well.

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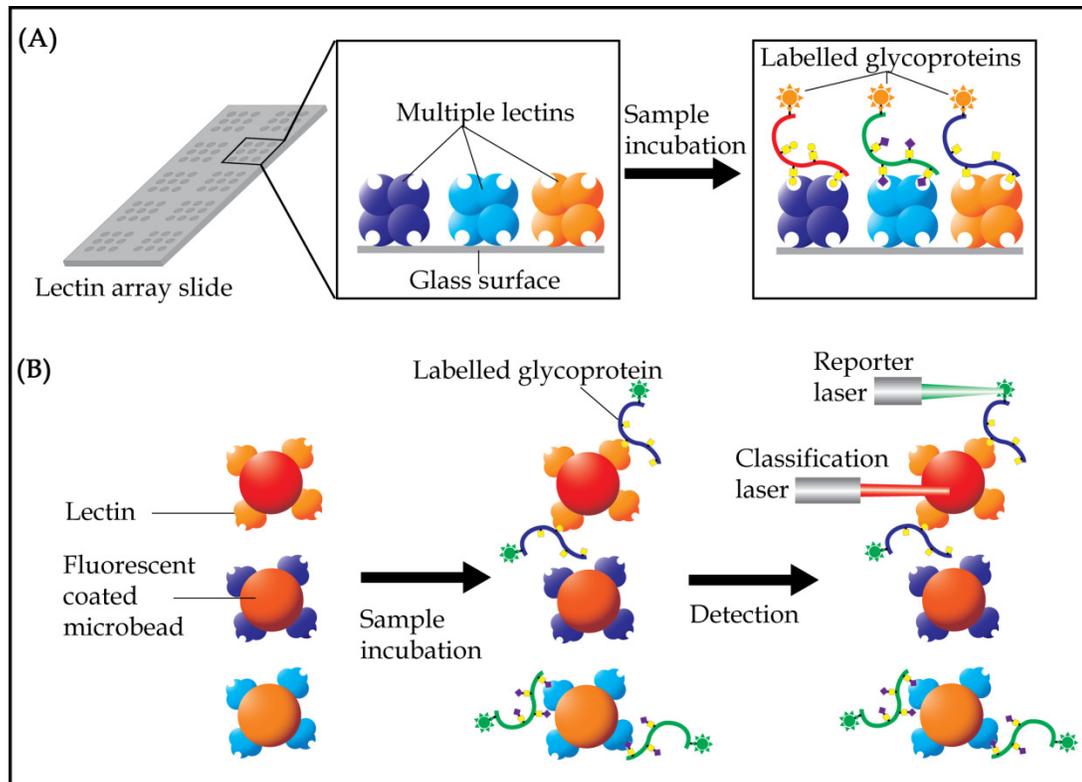
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458   **Figure 5**   **Basic concept of lectin array technology.** (A) Multiple lectins are printed onto a

459   slide, which is organized in a grid, single lectin per spot, format. Samples, which are usually

460   pre-labelled with either fluorophore or chromophore, are then allowed to interact with the

461   lectins. Lectin spots, which contain the labelled glycoproteins, will illuminate under an

462   appropriate scanner. (B) In lectin bead array analysis, different fluorescent colored beads,

463   each corresponding to a single lectin, are often used. The conjugated beads are then allowed

464   to interact with samples and the unbound materials being washed out. The beads are then

465   passed through a detector with two laser sources, with the classification laser identifying the

466   specific beads, whilst the reporter laser quantifying presence of the labelled samples.

467

468   By using an array of 45 different lectins to determine predictive biomarkers of colorectal

469   cancer, *Nakajima et al. (2015)* were able to identify 12 lectins that showed increase binding,

470   whilst 11 more lectins demonstrated low binding of glycoproteins in the colorectal cancer

471   tissues compared to normal epithelia. Amongst the lectins, *Agaricus bisporus* lectin which

472   was selected for further validation by the researchers, showed strong potential to be used as a

473   new predictive biomarker for distant recurrence of curatively resected colorectal cancer. A

474   similar approach performed on tissue extracts of gastric cancer demonstrated high

475   interactions of 13 lectins with tissue glycoproteins, whilst 11 others showed low interaction

476   (*Futsukaichi et al., 2015*). In both these studies, the altered interaction of lectins only

477   reflected the general presence of glycoproteins that were differently glycosylated without

478   providing any information on the precise glycoproteins that are affected.

479   In an earlier study, *Wu et al. (2012)* have used lectin array to screen for altered

480   fucosylated proteins in serum samples of patients with ovarian cancer. Based on the results,

481   the researchers then immobilized the lectins that showed differential interactions and used it

482   as affinity chromatography to isolate serum glycoproteins with aberrant glycan structures and

483   determine their protein identities. This strategy has led to the identification of four serum

484   glycoproteins with altered fucose residues. Recently, a different lectin array strategy was

485   also developed to serve as an analytical technique for determination of differences in

486   glycosylation of proteins that are isolated from serum samples (*Sunderic et al., 2016*). In this

487   study, the glycan content of serum alpha-2-macrogobulin, which was isolated from serum

488   samples of patients with colorectal cancer, was studied using the lectin array. From a set of

489   14 fluorescent labelled lectins that were used in the analysis, statistically significant

490   differences between two groups of patients with colorectal cancer and cancer negative

491   individuals were found for five of the lectins. When taken together, the results generally

492   showed that the alpha-2-macrogobulin of patients with colorectal cancer have higher content

493   of α2,6 sialic acid, GlcNAc and mannose residues, and tri-/tetraantennary complex type high-

494   mannose *N*-glycans.

495   Since its inception, the technology of lectin array has been through several modifications

496   to improve detectability of glycoproteins in biological samples. The array may involve prior

497   pre-capturing of a glycoprotein of interest using antibody, and the subsequent detection of

498   glycans using pre-labelled lectins (*Kuno et al., 2011*; *Li et al., 2011*). This approach allows

499   detection of the total glycan content of a specific glycoprotein and also reduces the need for

500   prior glycoprotein purification. Lectin array is not limited to glass slide as its solid support.

501   *Wang et al. (2014)* have used fluorescent dyes coated microbeads, which allows multiplex

502   detection in a single reaction vessel that greatly improves detection sensitivity compared to

503   the standard lectin arrays. More recently, an alternative approach which involves printing of

504   purified samples onto a chip surface has also been reported (*Sunderic et al., 2016*).

505   Lectin array analysis can also be performed on magnetic beads (Figure 5, panel B).

506   Known as lectin magnetic bead array, the technique was first introduced as a robust and high-

507   throughput pipeline for glycoproteomics-biomarker discovery in 2010 (*Loo et al., 2010*).

508   The method is based on use of multiple lectins that are conjugated to magnetic beads to

509   isolate glycan specific proteins. These lectin-conjugated beads are incubated with protein

510   samples, washed and the bound glycoproteins are then eluted in appropriate buffers for

511   subsequent proteomics analysis. By coupling a mass spectrometer to the one-step

512   glycoprotein separation and isolation procedure, profiling of glycan-specific proteins may be

513   achieved without much loss of proteins. This increases the probability of identification of

514   proteins of lower abundances that have biomarker potentials. Nevertheless, a few

515   methodological concerns need to be carefully considered when using the lectin bead array.

516   These include surface functionality and diameter of the beads, conditions of buffers and

517   duration of trypsin digestion protocols for optimal isolation of lectin-binding proteins. In this

518   technique, understanding of the specificities of lectins is also imperative as most glycosylated

519   proteins are expected to have multiple glycosylation sites for interaction with the lectins.

520   Using a panel of 20 lectins in a magnetic bead array that was coupled to a tandem mass

521   spectrometer, *Shah et al. (2015)* have demonstrated unique lectin-glycoprotein interactions in

522   serum samples that may be used to distinguish three groups of subjects comprising healthy

523   volunteers, patients with Barrett's esophagus and patients with esophageal adenocarcinoma.

524   Their results demonstrated the possibility of using apolipoprotein B-100 to distinguish

525   healthy volunteers from patients with Barrett's esophagus. The use of *Narcissus*

526   *pseudonarcissus* lectin in the assay was able to differentiate differently glycosylated

527   apolipoprotein B-100 in the two groups of subjects. On the other hand, patients with

528   Barrett's esophagus were markedly distinguishable from those with esophageal

529   adenocarcinoma via differences in the glycosylation of AAL-reactive complement

530   component C9, whilst PHA-reactive gelsolin was shown to have potential in differentiating

531   healthy subjects from patients with esophageal adenocarcinoma.

532

533   **Challenges and future directions**

534   Development and progression of cancer are associated with altered glycosylation and

535   aberrantly expressed glycoproteins. Hence, the use of lectin-based assays and strategies that

536   are discussed in this review article, together with the emergence of proteomics technology,

537   has led to identification of hundreds of putative glycopeptide biomarkers that can be utilized

538   in clinical practice. A summary on the advantages and disadvantages of these lectin-based

539   techniques is shown in Table 4. However, the translation of biomarkers from discovery to

540   clinically approved tests is still much to be desired. This is mainly attributed to the lack of

541   follow-up characterization and validation investigations of the potential biomarkers, which is

542   an absolute requirement to ensure that the discovery phase experiments are not flawed and

543   that detection of the biomarkers is reproducible, specific and sensitive (*Diamandis, 2012*;

544   *Drucker and Krapfenbauer, 2013*).   A potential glycopeptide biomarker has to be validated

545   using hundreds of specimens to become clinically approved tests. Hence, this is certainly not

546   possible in cases of rare cancers.

547

548   **Table 4** Advantages and disadvantages of lectin-based techniques in cancer biomarker
549   discovery research.

Techniques	Advantages	Disadvantages
Lectin affinity chromatography	•Does not require purified glycoproteins or glycans •Detailed analysis of glycan •High affinity	•Requires large amounts of samples •Time-consuming •Allows for individual samples only •Co-elution of other proteins
Enzyme-linked lectin assay (ELLA)	•Relatively high-throughput •Quantitative •Easy to perform •Very cost effective •Requires minute amounts of samples •In case of hybrid ELLA, prior	•Glycoproteins that are detected may not be identifiable unless it is coupled with further proteomics analysis or antibody detection. •In case of hybrid ELLA, non-specific direct interaction of

	purification of a glycoprotein is not required	lectin to antibodies may occur • Require purified glycans or glycoproteins as standard
Lectin histochemistry	• Simple • Rapid • Allows lectin multiplexing with the use of fluorescent tags	• Requires skills for tissue preparation • Requires use of multiple lectins/antibodies to provide further confirmation • Certain fixatives or components may reduce sensitivity
Lectin blotting	• Visualization of small amounts of proteins • Easy to detect • High specificity and sensitivity • Reliable and reproducible • Convenient method of screening of complex protein samples	• Choice of membrane may affect protein binding capacity and chemical stability
Lectin array	• Does not require purified glycoproteins or glycans • Rapid • Highly sensitive • High-throughput • Allows multiplexing • Requires small amounts of samples	• Requires extensive optimization • Possible non-specific interaction

550

551

552   In some cases, validation may not be successful with the use of a single cancer biomarker

553   in a single assay. One solution is to explore the simultaneously use of several different

554   biomarkers for development of a highly specific and sensitive assay (*Pang et al., 2010*).

555   Hence, there is an urgent need to consolidate data on availability of all putative glycopeptide

556   biomarkers that have been unmasked from the discovery phase studies for every different

557   application in every cancer. In addition, new high throughput assays for simultaneous

558   detection of multiple biomarkers are also required. The recent technological advances in

559   chip-based protein microarray technology (*Sauer, 2017*) may provide with the solution, and

560 therefore ought to be explored for simultaneous validation analysis of the different

561 biomarkers in a single experiment.

562 In many other cases, identification of the potential glycopeptide biomarkers using lectin-

563 based strategies may involve complex separation techniques such as 2-DE, which is

564 laborious and expensive for large scale validation studies. 2-DE comes with the advantage of

565 knowing the actual experimental molecular weight of a glycopeptide biomarker, which is not

566 possibly attained from liquid-based separation methods. This is important as many tumor

567 associated glycopeptides are known to be truncated products of native glycoproteins (*Pinho*

568 *and Reis, 2015*). For these potential biomarkers, validation experiments would need to

569 involve a different indirect high-throughput technique using both lectin as well as an

570 antibody that is capable of differentiating truncated glycopeptides from their native

571 glycoprotein structures. However, such antibodies are usually not available commercially,

572 and generating them is time consuming, costly and involves substantial laboratory work.

573

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