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Network medicine in drug design: implications for neuroinflammation

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Neuroinflammation is a general innate defensive response to neurotropic pathogens, neurodegenerative diseases or brain injuries, brought about by active proinflammatory signaling by the glial cells (microglia and astrocytes). Because these inflammatory signaling pathways cross-talk with each other, drug targeting at any particular intermediate molecule is not effective. Network medicine is a network theory inspired approach in drug design, whereby various mathematical models are applied to identify plausible nodes within a signaling pathway simulated network important for drug targeting. There are many techniques involved in network medicine study; in this article we concentrate on the 'prioritization of protein clusters' responsible for a certain disorder. This approach aims to bring down the expenditure of resources of initial drug targeting against a complex pathological reaction, such as neuroinflammation, and also questions the cause at the molecular level.

The brain has long been termed as the 'immune privileged' organ, because it is isolated from the peripheral system by the blood–brain barrier (BBB) and it prevents the infiltration of the leukocytes. The idea has been debated since 1995 owing to evidence of innate immune responses by the cells of the central nervous system (CNS) [1]. The phenomenon of the innate immune response of the brain brought about by brain injury, stroke, neurotrophic pathogens or other sources is termed as 'neuroinflammation'. The acute version of inflammation of the brain is referred to as 'reactive gliosis'. This is observed when there are minor insults to the brain, where accumulation of enlarged glial cells, such as microglia and astrocytes, occurs and factors which target and stimulate the target cells to produce inflammatory responses are released. Chronic inflammation is brought about by sustained cycles of injury and cumulative ill effects of immunological microglial and astrocytic activation which is responsible for focal loss of brain functions. Neuroinflammation is more pertinent in CNS diseases, thus understanding the cause of the basic neuroinflammatory mechanism is important for drug targeting [2].

Neuroinflammation mainly involves two types of immune cells: lymphocytes, monocytes and macrophages of the hematopoietic system, and microglial cells of the CNS [3,4]. These immune cells remove the cell debris and release a host of powerful regulatory substances, for example, complements, cytokines, chemokines, glutamate, interleukins, nitric oxide, reactive oxygen species (ROS) and transforming growth factors (TGFs) [5–9]. These substances act as double-edged swords for the cellular environment in brain. By contrast, astrogliosis has been reported for anti-inflammatory activities, repairing the BBB and attenuating further neuronal death [4,10]. Neuroinflammation has been documented in several cases of neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), depression, epilepsy, Huntington's disease (HD), multiple sclerosis (MS), Parkinson's disease (PD) and viral encephalitis [8,11–13].

Neuroinflammation: where all neurodegenerative signaling pathways converge

As mentioned in the previous section neuroinflammation is a common pathophysiology for most of the neurodegenerative diseases; through various sources they activate the glial cells (especially microglia) which on activation stimulate the production of inflammatory mediators, such as cytokines and chemokines, resulting in neuroinflammation. The pathways through which various cytokines and chemokines produced are not definitive for all diseases, and depends mostly on the receptor which helps to activate the microglia [14].

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Activation of microglia, often referred to as the converging point in most of the neurodegenerative disease sequel, has been evidenced from epidemiological, pharmacological and genetic analysis. In normal physiological conditions, microglia express surface receptors, such as CD (cluster of differentiation)-45, CD-14 and CD11b/CD-18 (Mac-1) [15]. It has been found that in the activated state microglia express surface receptors, such as CD1, lymphocyte function-associated antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1 or CD-54) and vascular cell adhesion molecule-1 (VCAM-1 or CD-106). In addition, mediators including cytokines [TNF (tumor necrosis factor)-α, IL (interleukin)-6, IL-1β, among others] and chemokines [MCP-1 (monocyte chemoattractant protein-1) and IFN (Interferon)] are secreted. Furthermore, ROS and inducible nitric oxide synthetases (iNOS) are released, which are also potent mediators of inflammatory response [15].

AD is characterized by the mutation of amyloid precursor proteins (APPs) or components of its machinery (α- and γ-secretase) resulting in over production of Aβ 1–40 and 1–42 peptides, which are reported to be the potential cause of the disease [16–22]. Microglia is found to be activated near the amyloid plaque formation and upregulates the human leukocyte antigen-DR (HLA-DR) [23]. Aβ deposition is reported to increase the release of cytokines, such as TNF-α, IL-6 and IL-1β from the microglia near the plaque formation, which has been implicated in inhibiting the phagocytosis of Aβ by microglia [24,25].

In the case of a neurotropic viral infection, there are evidences of the involvement of neuroinflammation and microglial activation. In Japanese Encephalitis Virus, it has been reported that there is a multiform rise in proinflammatory mediators like iNOS, Cox-2 (Cyclooxygenase-2), IL-1β, IL-6, TNF-α and MCP-1 post-infection and maximized secretion in hippocampus, indicating the activation of microglia in the same region [26]. The major signaling pathways in microglia for stimulating inflammatory response are nuclear factor kappa beta (NF-κB) pathway and p38-mitogen activated protein kinase (MAPK) pathway [27,28]. Both have been reported to regulate the transcriptional machinery of proinflammatory mediators once they have been stimulated by specific receptors on the microglial cell surface. Both NF-κB and p38-MAPK are activated by signaling receptors, such as Toll-like receptor (TLR), retinoic acid-inducible gene I (RIG-I) [29], interleukin-1 receptor (IL-1R) and tumor necrosis factor receptor (TNFR) [30,31]. Depending upon the activating signal, each follows either an independent pathway or cross-talk to bring about the transcription of proinflammatory mediators in the nucleus. Because these major pathways are the main routes for inflammation, drug targeting specific proteins of these pathways can be effective to hinder inflammatory responses.

Network medicine: futuristic approach in drug targeting

The concept of network medicine is summarized by the phrase 'think globally, act locally' [32]. This approach of drug targeting intervenes in local protein–protein interactions (PPIs), promoter–transcriptional factor interactions, post-translational modifications (PTMs) and metabolic reactions which determine cell fate for migration, proliferation, apoptosis, or differentiation to bring on global effect in the physiological environment [33].

The introduction of computational algorithms in bioscience research has changed the facade of drug discovery and development industry. In particular to drug targeting in neuroinflammation research, the previous trend was to target a particular molecule in the neuroinflammatory pathway with a drug depending upon its molecular structure, not considering the effect of inhibition or activation of that particular molecule in the global environment of the cell. Moreover, it did not consider the effect that the drug might have on other molecules within the cell with which it might interact and cause side effects, nor did it consider the neuron–glial interactions at cellular level. Today, most drugs are developed in highly specialized biotechnology companies [34]. It has been known that the hike in drug development price is not owing to an increase in clinical trial time, because the review process time has declined [35]. It has been debated that modern drug industries favor funding 'non-risky drug development' to produce well-studied drug targets [36].

Network medicine approach mainly relies on two aspects: (i) detailed understanding of the molecular mechanisms including their dynamics and specific binding domains; (ii) formulation of effective computational algorithm to model the signaling events. The synthetic biology approach to understand the network nature of the inflammation related signaling pathways can be designed in two ways. Firstly, exploiting the modular nature of the signaling proteins to modify the topology and rewiring of the network by adding and depleting interactions [37]. Secondly, considering the dynamic nature of the signaling pathways, molecular modifications (*e.g.* phosphorylation and dephosphorylation) are modeled to proceed in a hierarchal manner adding control architectures at each step. This explains that drug targeting of the inflammatory pathways can be brought about by targeting phosphorylation states of the key proteins in the information flow within the network.

There are plethora of molecular modification mechanisms involved in neuroinflammatory cell signaling, but recently researchers are preferring to pin down phosphorylation events which mainly governs the cellular information processing that controls PPIs, complex formation, enzyme activity, protein degradation and translocation [38]. The developments in mass spectroscopy (MS) have come up with identification and quantification of several *in vivo* protein phosphorylation sites. Among these are phospho-flow [39,40], kinase-activity assays and conventional immunoblotting.

The principle of phospho-flow technology is based on the detection of phosphorylating event within a cell by a specific antibody. In this technique, initially the cells are labeled by antibodies to recognize specific phosphorylated proteins and cell markers. Thereafter they are sorted by fluorescence activated cell sorting (FACS) to provide information about quantitative measurement of single-cell signaling events. By contrast kinase assays of various types provide firsthand information about the enzyme activities by kinase-specific purification or specific kinase chemosensor [41,42]. MS is a potential approach to analyze protein networks [41–47], along with the quantification of protein in addition to analysis of PTM (identifying dynamic changes in proteins, such as phosphorylation) [45–49] and protein dynamics. Other techniques, such as stable isotope labeling with amino acids in cell culture (SILAC) (incorporating stable isotopes into proteins in cell culture) [47], isobaric tags for relative and absolute quantitation (iTRAQ) (enabling four to eight channel

multiplexed experiments in which different samples can be combined) [47,49] are being used for identifying relative amounts of proteins between the network states sampled and finding out their binding partners, and quantifying proteins across different experimental conditions, respectively. Thus together, PTM, SILAC and iTRAQ are important protein quantification tools of wet laboratories, which can give a robust analysis of proteins involved in neuroinflammation.

The data obtained through various molecular techniques mentioned above is modeled by a combination of partial least square regression (PLSR) for numerical modeling and principal component analysis (PCA) for data condensation. It has been reported that cytokine-induced apoptosis, which is a crucial event in neuroinflammation, has been modeled through PLSR [50]. Recently, there has been report that clusters the various proteomic data available from a dynamic phosphorylating analysis with the help of statistical metadata, and tries to infer about the functional meaning of various phosphorylating states of proteins in a cell signaling network [51].

Barabasi *et al.* took up a networking approach known as 'Diseasome Bipartite Model' which is a bipartite graph involving two disjoint sets of nodes. Of which one enumerates the known genetic disorders, whereas the other comprises of the disease genes in the human genome (Fig. 1). From the figure it becomes clear how the relationship between genetic mutations, followed by malfunctioning of the signaling pathways is related to various genetic disorders. It can also be inferred that the causality of a particular genetic disorder is not only owing to mutation at a particular gene, but involves a cluster of malfunctioning genes [33]. This view suggested the 'hub' concept in case of complex diseases (*e.g.* neuroinflammation). Hence all these various analytical approaches empower the building of a solid foundation to the network modeling.

Prioritization concept: a mathematical tool for network medicine

The concept of network medicine is supported by both biological analysis and their mathematical modeling. With the developing

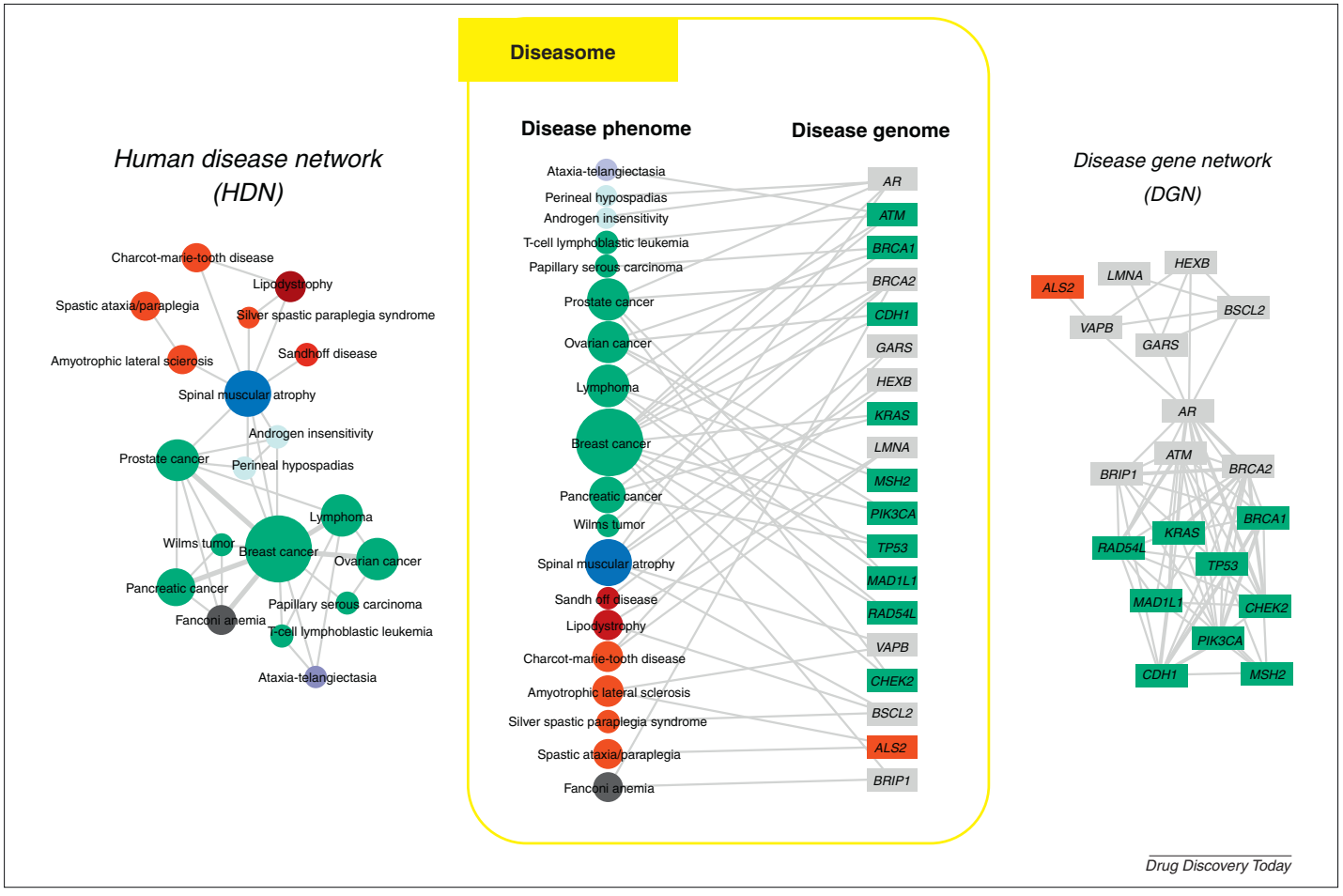

FIGURE 1

Diseasome Bipartite Model. The diseasome section in the center of the figure represents a small subset of OMIM based disease–disease–gene associations, where circles represent the diseases and rectangles represent the gene. Each link connects the mutated gene and its corresponding disease. The size of the disease circle increases with the increasing association with the mutated genes, the color represents the class of disease. On the left, human disease network (HDN) shows the various genetic diseases in human links connecting diseases that share similar gene mutations. The width of link increases with the number of associated genes between each disease pair. On the right, disease gene network (DGN) shows that two genes are connected if they are involved in same disease. The width of link similarly increases with the number of diseases each gene pair is associated with.
(Adapted from [33]).

concept of the 'omic' studies, people are able to gather knowledge about various gene–protein–phenotype interactions in cases of various diseases. From previous drug design theories, it became difficult to target a single gene, because it has been known that a particular gene is not singularly responsible for a certain disorder. This lead to a search for all the genes those are directly or indirectly responsible for a particular disorder or more specifically a particular phenotype corresponding to that disorder [52]. Thus this approach can be a useful application in neuroinflammation drug design.

From linkage analysis studies gene loci connected to a particular disease can be known (*e.g.* lying between genomic intervals of 0.5–10 cM) but it might contain a set of 300 genes in total [53,54]. Thus, in that case identifying the causal genes for a particular disorder (*e.g.* neurodegenerative diseases) becomes difficult. Hence a concept of gene–protein–phenotype network has come up that tries to prioritize protein clusters by providing ranks, involved in that particular disorder to build a hierarchy of causal genes, and hence suitable drug targets. This concept of prioritization is modeled by various mathematical approaches.

The first theory that was put into was of incorporation of Bayesian Predictor, whose scoring pattern of the protein clusters was based on the Bayes' Theorem. A brief idea of the entire system can be visualized in Fig. 2. The phenotypic overlapping was quantified by a scoring system designed by Lage *et al.* [52].

The Bayesian Predictor from the high confidence data scores and ranks them accordingly by applying the Bayes' Theorem. The model includes parameter for (i) probability of candidate protein has any known interaction partners, (ii) protein interaction score, (iii) the number of interaction partners involved in similar disorders and (iv) computational phenotype similarity score (derived from Ref. [52]).

The Bayesian Predictor model is given as:

$$P(\text{dis} = i|\text{DATA}) = \frac{P(\text{DATA}|\text{dis} = i) \times P(\text{dis} = i)}{\sum_{j=1}^{n} P(\text{DATA}|\text{dis} = j) \times P(\text{dis} = j)}$$

where i represents protein number (among n candidates) associated with disease, $P(\text{dis} = i|\text{DATA})$ is the posterior probability that the protein i is the disease-related one after evaluating all the data. $P(\text{dis} = i)$ represents the prior probability of the candidate number i is the disease-causing protein, before evaluating any data. The prior value for all candidates needs to be set at $1/N$ at beginning. $P(\text{DATA}|\text{dis} = i)$ denotes the probability of obtaining the observed data if candidate number i is indeed the correct one. The likelihood is computed from analyzing interaction data and associated phenotype description, and utilizing the estimated paramaters. The

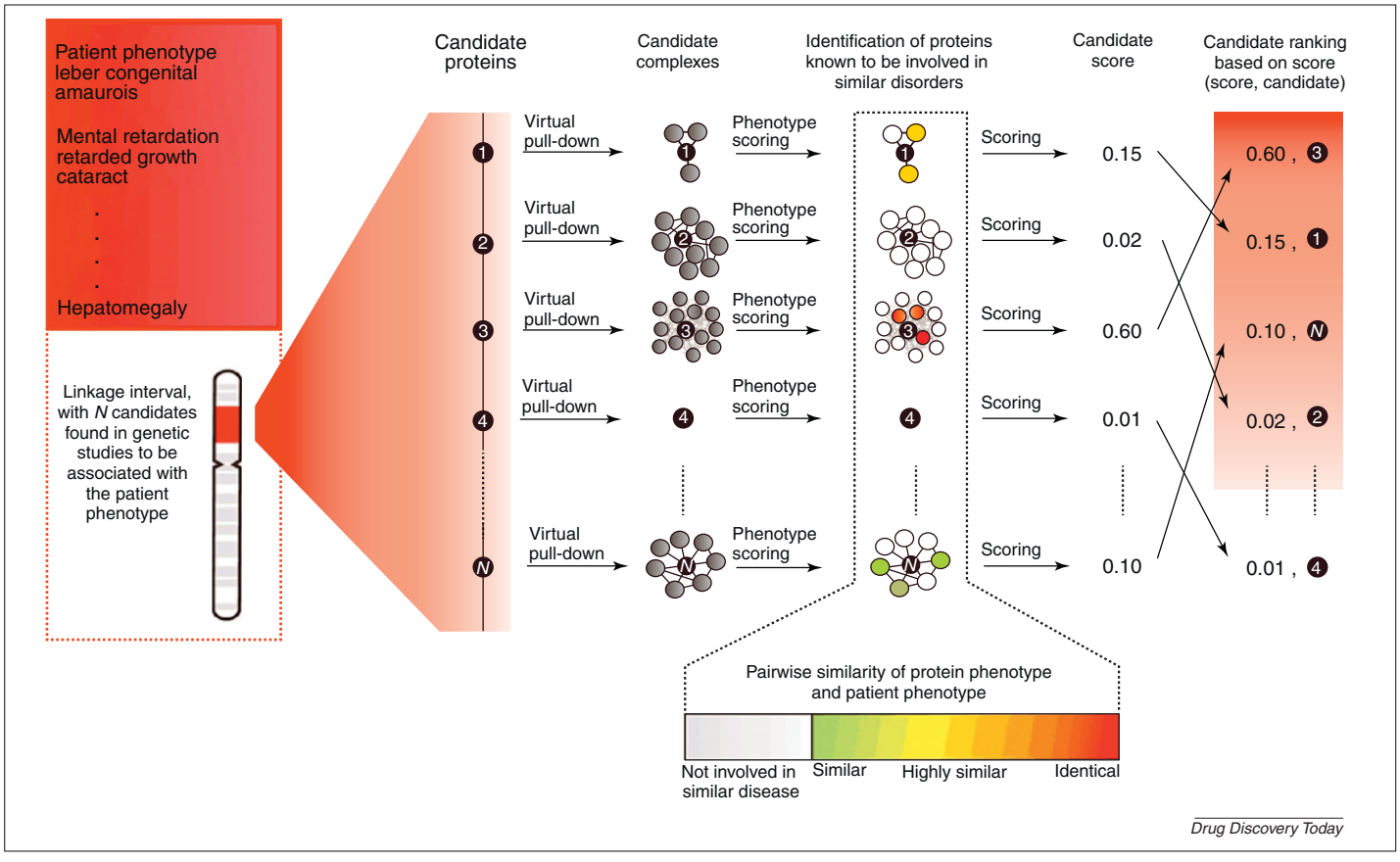

FIGURE 2

Algorithm for scoring candidate proteins in a linkage interval. In the first step there is a virtual pull down of all the candidate proteins involved in a particular disorder. Each complex is named as the candidate complex. In the second step, proteins involved in disorder are identified from the candidate complex, and the pairwise similarity is determined by text mining. The similarity is determined from the color code shown in the figure. In the concluding step, the scoring and ranking of the candidates are done by applying the Bayesian Predictor.
[Adapted with permission from Macmillan Publishers Ltd.: (Nat. Biotechnol.) [52]].

denominator probabilities mean the same as those for the numerators but for all the protein candidates irrespective of their disease associations [52].

The Bayesian Predictor concentrated more on local PPI associated in a disease. The methods that came up after this were more related to study on global perspective of PPI. The Walking Interactome Model was one among those, designed by Kohler *et al.* [55]. This theory was based on graph theory, walking through the shortest path of PPI. PPI network is built on the fact that genes are denoted by nodes and the PPI interactions by edges. The scoring system adapted in this case is based on random walk and diffusion kernel, thereafter fixing the ranks of the candidate proteins. In random walk, the probability of being at node i at time step t is calculates as vector $\mathbf{p}^t$:

$$\mathbf{p}^{t+1} = (1-r)W\mathbf{p}^t + r\mathbf{p}^0$$

The walking includes transition from one node to the other starting from a source node, s requires to restart its walking whenever $\mathbf{p}^t$ and $\mathbf{p}^{t+1}$ (measured by L_1 norm) fell below 10^{-6}, with a probability r. W in the above equation denotes the column-normalized adjacency matrix of the graph. Initial probability vector is given as $\mathbf{p}^0$ to nodes representing members of the disease, cumulating all probabilities to 1, whereas the steady state probability is represented by $\mathbf{p}\infty$ [55].

In diffusion kernel scoring, the summation of diffusion kernel K of a graph is taken as $K = e^{-\beta L}$, where β controls the magnitude of diffusion. Matrix L is the Laplacian of the graph, which is equal to the difference $D - A$, where A is the adjacency matrix of interaction graph and D is a diagonal matrix containing the nodes' degree (Fig. 3). The score of candidate gene i given as

$$\text{score}(j) = \sum_{i\,\varepsilon\,\text{disease gene family}} Kij$$

In some recent literature there has been mention about the efficiency of this Random Walking Method, which in a few ways is better applicable than even its successors. Also its applicability and efficiency in predicting gene–disease associations can be enhanced by combining it with modern clustering methods [56].

Correlating protein interaction network and phenotype network to predict disease genes (CIPHER) is another prioritization technique designed by Wu *et al.* [57] keeping in the global PPI in view like the Walking Interactome Model discussed above. CIPHER is advancement in Walking Interactome Model in the respect that the scoring system it follows is based on the linear regression model. This model calculates the correlation between the closeness profile of the candidate genes (g) and similarity profile of the query phenotypes (p) and assigns a score to the candidate gene. Hence, thereafter ranking is done on the basis of

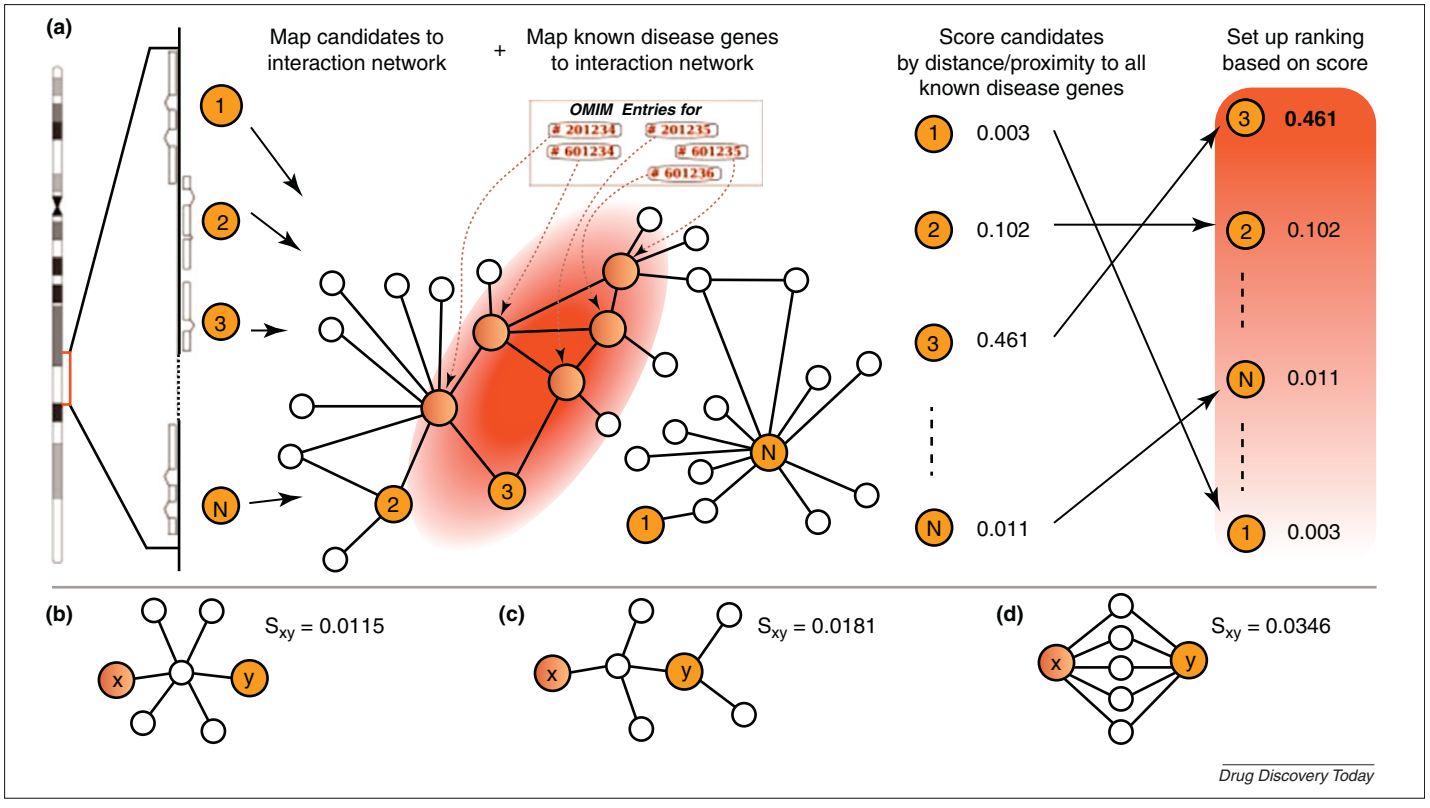

FIGURE 3

Disease–gene prioritization by Random Walking Process. **(a)** Describes the procedure followed in Walking Interactome Model for disease–gene prioritization. The initial method involves identifying the linked genes, and then from those finding the genes linked to a particular disease and the candidate protein by virtual pull down. Thereafter an entire network of genes and protein–protein interaction (PPI) is built. Scoring is given with the application of distance and/or proximity to all known disease genes by Random Walking and Diffusion Kernel methods. Hence ranks are assigned to the candidate proteins. **(b)–(d)** various forms of interactions are represented, where b, c, d represents 'hub' model, fewer connections than hub model, mutual interactive model, respectively where x and y are disease gene and candidate gene, respectively. s_{xy} represents the similarity score between the two. The more the interactive connections or in a way the more precise the connectivity the higher is the similarity score.
(Reproduced from [55]).

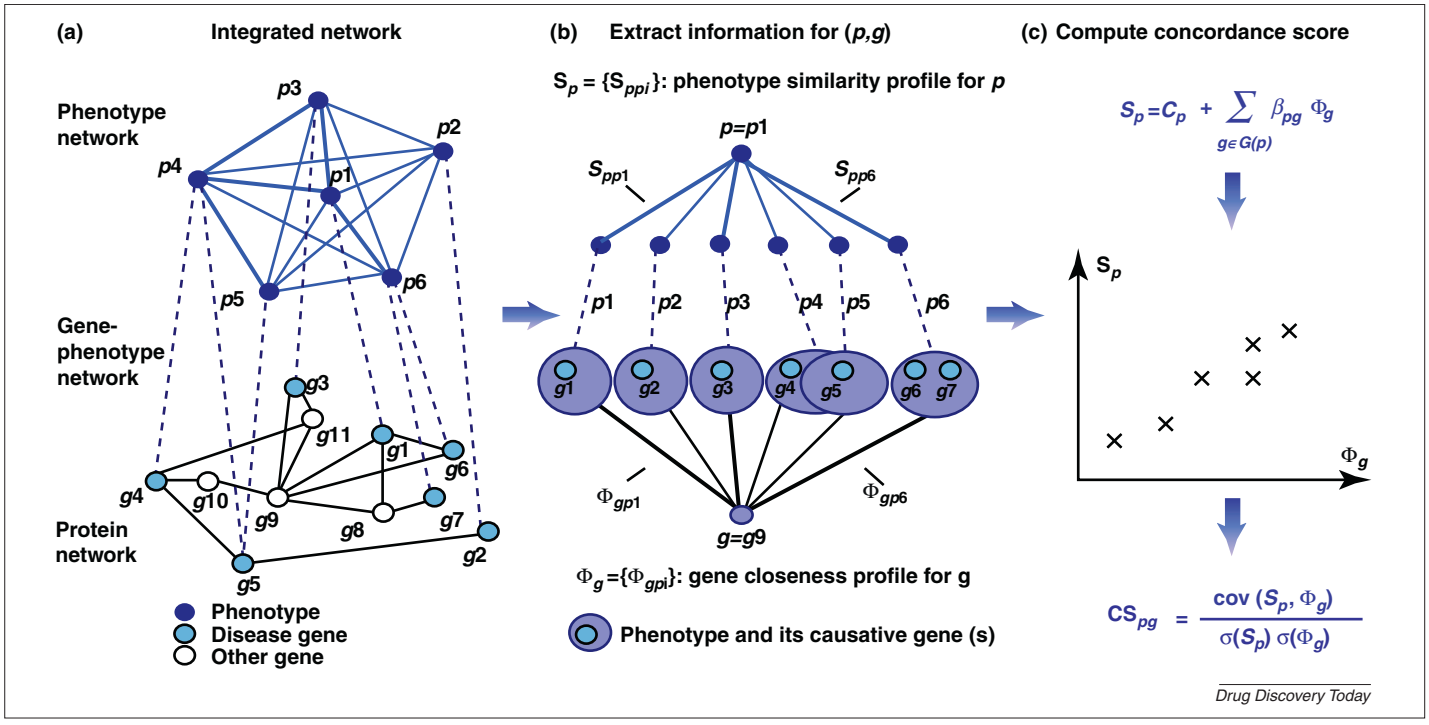

FIGURE 4

Algorithm for scoring candidate proteins in CIPHER method of prioritization. Describes the formulation of CIPHER wherein **(a)**, phenotype network, gene–phenotype network and protein network are represented into an integrated network. **(b)** The two profiles: closeness profile computed in genes and similarity profiles extracted in phenotypes from the integrated network. **(c)** The concordance score is assigned by calculating the correlation between the two profiles of phenotype (p) and gene (g). Where s = similarity profile, s_p = similarity profile for phenotype, s_{ppi} = similarity profile for phenotype interaction of ith cluster, ϕ = gene closeness profile, ϕ_g = gene closeness profile for a gene, ϕ_{gpi} = gene closeness profile for gene–phenotype interaction of ith cluster, C_p = being a constant, $G(p)$ = denotes all disease genes related to a particular phenotype, β_{pg} = denotes level of gene g contributing to a similarity of phenotype p to another p', CS_{pg} = concordance score for a phenotype related to a gene, cov = covariance, σ = standard deviation.
(Adapted from [57]).

the score. The entire model is described in Fig. 4. Because two main forms of topological distances are involved CIPHER has been classified into two profiles; CIPHER is classified into CIPHER-SP and CIPHER-DN (SP: shortest path and DN: direct neighbor). The closeness profile in the modeling is represented by:

$$\phi_{g\,p'} = \sum_{g' \varepsilon G(p')} e^{-L^2_{gg'}}$$

where p' refers to other phenotype apart from p and g' refers to any other gene apart from g. $L_{gg'}$ refers to topological distance between g and g' [57]. The similarity profile is represented as:

$$S_{p\,p'} = C_p + \sum_{g \varepsilon G(p)} \beta_{pg} \phi_{g\,p'}$$

where β_{pg} refers to the coefficient of regression model a contribution factor of gene g to a particular phenotype similarity between p and p', whereas C_p is a constant [57].

The correlation is calculated by Pearson linear correlation coefficient as concordance score:

$$CS_{pg} = \frac{\mathrm{cov}(S_{p'} \phi_g)}{\sigma(S_p)\sigma(\phi_g)}$$

where cov and σ mean covariance and standard deviation, respectively. The correlation between the similarity and closeness shows how the closeness of two genes varies with the similarity between the corresponding two phenotypes (Fig. 4). In special cases where genes do not link to any disease the $\phi_g = 0$, for that CS_{pg} cannot be computed. In such cases $CS_{pg} = -1$, and hence those genes are ranked last [57].

From the above discussion we can come to few conclusions: that Bayesian Predictor concentrates more on local PPI whereas Walking Interactome and CIPHER models masters in their prioritization approach in global environment of PPI. However, in isolation it implies that a gross mechanism is lacking for an efficient drug targeting.

Vanunu *et al.* came up with a new model known as prioritization and complex elucidation (PRINCE) which tackles both the challenges to some extent and promises an effective drug targeting model. The model uses the iterative propagation based algorithm that makes the work of prioritization faster with more precision. The iterative model works as:

$$F^t = \alpha W^{F^{t-1}} + (1 - \alpha) Y$$

where F represents the prioritization score, and Y denotes the prior information about a PPI interaction. α controls the relative importance of prior information in association assignment whereas W' are eigenvalues. The entire equation through iterative steps pumps in information from the candidate nodes to their neighboring nodes. Y is calculated from a logistic function that is derived empirically. Thus from a given disease–disease relationship and finding out the prioritization score for the network proteins, a kind of filtering is done in this method to select the more relevant

protein complexes involved in a particular disease. For example, initially all those candidate proteins will be eliminated that are below a certain threshold score. Thereafter as a process of continual refinement, it will gradually filter out candidate proteins keeping only those which are most interactive and highly significant to be declared as causal for a particular disease [58].

To summarize protein prioritization techniques help to fish out the potential therapeutic targets from the complex network of neuroinflammatory cellular signaling pathways. By applying modern bioinformatics techniques the structure of these proteins will be figured out to identify their ligand binding sites. Performing docking studies molecules specifically binding to those binding sites are to be designed. Thereafter screening of the molecules on the basis of available databases is carried out along with few downstream processing for the purpose of drug design [59].

Concluding remarks

Neuroinflammation is a common pathophysiological condition of various neurodegenerative diseases; targeting the gene and protein interactome model involved in it is a potential approach of drug targeting. Thus, by targeting the genes and the proteins involved in neuroinflammatory pathways we can find a cure to a spectrum of neurodegenerative diseases along with neurological disorder brought about by neurotropic pathogens, stroke among others.

We have come across various molecular techniques that are being applied to understand the dynamics and specificity of the molecular reactions involved in the interacting signaling pathways. Although the available techniques are helping to get closer to the *in vitro* cellular conditions and bring out robust information, however, they have various constraints. For example, as mentioned in the previous text, various techniques are trying to target the phosphorylating sites in the information flow of signaling pathways. However, there are other reaction mechanisms that need to be addressed. The phospho-flow techniques could be efficient in picking up about detailed phosphorylating events, but it is not catering to other reactions which might come forward with more reasonable answers. Hence, new integrated methods are needed to be developed to formulate a strong understanding of the problem.

Statistical data management is the next problem that the network medicine technology is facing. Existing techniques, such as PCA and PLSR which have been used till now for this kind of research have their own constraints. PCA being a linear algorithm fails to classify real world data in an accurate manner because real world data are mostly non-linear in nature. Moreover PCA needs to do a tradeoff between maximizing variance and maximizing information. Last but not the least, the prioritization models discussed requires going a few more steps to present a holistic representation of the *in vitro* events. Although the PRINCE technique claims to be a more futuristic model than its predecessors like Random Walking and CIPHER, it is still far away from the wet laboratory experimental results.

Keeping in mind the above limitations, we have to appreciate the approach of network medicine towards solving the mystery of complex diseases and finding potential drug targets. The initial efforts must be given to study the various neuroinflammatory pathways, and come out with inferences about principle molecules involved in these. With a developed prioritization model these principle molecules or the genes giving rise to them can be prioritized for being selected as drug targets. Thereafter by analyzing their structural make-up and finding proper binding sites drug development can be done. Network medicine promises not only a cure for complex diseases (*e.g.* neuroinflammation) but also to minimize the cost invested by the drug developing industries in drug targeting experiments based upon the 'hit and trial method'.

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