Dear RECOMB Program Committee members and Genome Research editors,

Please find enclosed a manuscript entitled "Positional gene enrichment analysis of gene sets for high resolution identification of overrepresented chromosomal regions" co-authored by Katleen De Preter, Roland Barriot, Frank Speleman, Jo Vandesompele and Yves Moreau.

The paper describes a new method to identify chromosome regions significantly enriched in a given set of genes, and its application on published gene lists of differentially expressed genes in human B-CLL leukemia, neuroblastoma tumors and tissues from Down syndrome patients.

The complete address of the corresponding author is:

Roland Barriot
ESAT-SCD, Katholieke Universiteit Leuven
Kasteelpark Arenberg, 10
B-3001 Leuven
Belgium
tel.: +32 16 32 86 50
fax.: +32 16 32 19 70
e-mail: Roland.Barriot@esat.kuleuven.be

We hope that you will find our paper suitable both for a RECOMB oral presentation and for publication in Genome Research.

Sincerely,
Roland Barriot.

Positional gene enrichment analysis of gene sets for high resolution identification of overrepresented chromosomal regions

Katleen De Preter[1,*], Roland Barriot[2,*], Frank Speleman[1], Jo Vandesompele[1], Yves Moreau[2]

[1]Center for Medical Genetics, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

[2]ESAT-SCD, Katholieke Universiteit Leuven, Kasteelpark Arenberg 10, B-3001 Leuven, Belgium

*These authors have contributed equally to this work and should be considered shared first author.

Corresponding author: Roland Barriot, Roland.Barriot@esat.kuleuven.be

Abstract

The search for feature enrichment is now a widely used method to characterize a set of genes or proteins. While several tools have been designed and are available for nominal features such as the Gene Ontology annotations or the KEGG Pathways, very little has been proposed to tackle numerical features such as the gene coordinates on the chromosomes. For instance, microarray studies typically generate lists of genes that are differentially expressed in the sample subgroups under investigation, and when studying diseases caused by genome alterations such as cancer, it is of great interest to delineate the chromosomal regions that are significantly enriched in differentially expressed genes. In this article, we present a positional gene enrichment analysis method for the identification of chromosomal regions that are significantly enriched in a given set of genes. The strength of our method relies (i) on an original query optimization approach that allows to virtually consider all the possible chromosomal regions for enrichment, and (ii) on the correction applied in the context of multiple testing which discriminates truly enriched regions versus those that can occur by chance. We have developed a Web tool, **PGE** (positional gene enrichment), implementing this method applied to the human genome. PGE allows one to submit a set of gene identifiers and visualize enriched regions. For validation, we used PGE on published lists of differentially expressed genes observed in B-cell chronic lymphocytic leukemia, neuroblastoma tumors and tissues of Down syndrome patients. These analyses showed significant overrepresentation of known aberrant chromosomal regions.

Availability: PGE is publicly accessible at `http://www.esat.kuleuven.be/~bioiuser/pge`

Keywords: gene enrichment, gene sets, expression microarrays, genome aberration

Introduction

Microarrays are powerful tools to study gene expression patterns on a genome wide scale. From the raw microarray data, various gene lists are sifted out based on their differential expression in the subgroups under investigation. These gene lists provide the foundation from which to begin the exploration of the underlying cellular biology resulting in the observed phenotype. A data-mining approach that is increasingly used for study of such gene lists is the search for enriched terms associated with the individual genes. Instead of focusing on the actual genes, such analyses try to summarize the information using annotated and structured information, such as hierarchically organized Gene Ontology (GO) annotation terms and KEGG cellular pathways. For enrichment analysis of the latter two gene classifications, many software tools are available (Barriot, Poix, Groppi, Barre, Goffard, Sherman, Dutour and de Daruvar 2004; Khatri, Draghici, Ostermeier and Krawetz 2002; Zhang, Schmoyer, Kirov and Snoddy 2004).

Another -less frequently explored- gene characteristic for the study of microarray gene lists is the chromosomal position of the genes, especially when studying diseases caused by genome alterations, such as cancer. Exploration of the relationship between gene copy number alterations and gene expression in breast tumors for instance revealed that a high percentage of amplified genes were overexpressed (Buness, Kuner, Ruschhaupt, Poustka, Sultmann and Tresch 2007; Hyman, Kauraniemi, Hautaniemi, Wolf, Mousses, Rozenblum, Ringner, Sauter, Monni, Elkahloun et al. 2002; Pollack, Sorlie, Perou, Rees, Jeffrey, Lonning, Tibshirani, Botstein, Borresen-Dale and Brown 2002). Other studies showed systematic upregulation of many genes on chromosome 21 in Down syndrome patients, harboring an extra copy of this chromosome in all their cells (Kahlem 2006). In acute myeloid leukemia DNA gains and losses caused by multiple chromosome rearrangements were shown to result in altered gene expression in a gene-dosage-dependent manner (Lindvall, Furge, Bjorkholm, Guo, Haab, Blennow, Nordenskjold and Teh 2004). Comparable to the GO and KEGG pathway analysis tools, it would be of great interest to explore the overrepresentation of chromosomal regions in the generated microarray gene lists. Currently, various tools exist that identify entire chromosomes that are overrepresented in the gene lists (Hosack, Dennis, Sherman, Lane and Lempicki 2003; Khatri, Draghici, Ostermeier and Krawetz 2002; Zhang, Kirov and Snoddy 2005). However, the resolution is not sufficient enough to pinpoint critical dosage sensitive regions. Other tools allow the identification of overrepresented regions along the chromosomes in higher detail, such as expression imbalance maps (Kano, Nishimura, Ishikawa, Tsutsumi, Hirota, Hirose and Aburatani 2003), ChARM (Myers, Dunham, Kung and Troyanskaya 2004), MACAT (Toedling, Schmeier, Heinig, Georgi and Roepcke 2005) and DIGMAP (Yi, Mirosevich, Shyr, Matusik and George 2005). The

downside is that these methods need the microarray data as input, which is not always possible (*e.g.* raw data not made available) or more importantly because it restricts the search of enriched chromosomal region to gene expression and prevents its application to a given set of genes of interest. More recently, ChroCoLoc was developed and accepts a given list of genes as input (Blake, Schwager, Kapushesky and Brazma 2006). However, the resolution is limited to chromosomal bands *i.e.* only a fixed set of chromosomal regions are tested for enrichment.

Here, we present an innovative algorithm and accompanying software -accessible through an online Web interface- for positional gene enrichment (PGE) analysis.

Methods

Statistics

To test if a chromosomal region is enriched in a query set of genes of interest (for example differentially expressed genes), we apply the hypergeometric distribution as follows: Let *g* be the total number of genes considered (**g**enome), *t* the number of genes in the region (**t**arget set), *q* the number of genes of interest (**q**uery set) and *c* the number of genes of interest in the region (genes **c**ommon to the query and target set), then we have $\text{p - value}(c,t,q,g) = \sum_{k=c}^{\min(q,t)} \frac{\binom{t}{k}\binom{g-t}{q-k}}{\binom{g}{q}}$. This corresponds to the probability of having at least the observed number of genes of interest in that region. To correct for multiple testing (a large number of regions are tested), adjusted *p-values* are calculated using the minimum *p-values* cumulative distribution function. This function provides the probability of obtaining a *p-value* at least as good (lower or equal) by chance *i.e.* by submitting a random set of the same size. Unfortunately, it is practically impossible to model this distribution, so we approximate it by sampling to obtain an empirical function as describe in (Barriot, Poix, Groppi, Barre, Goffard, Sherman, Dutour and de Daruvar 2004) and (Dufour 1995).

Identification of pertinent chromosomal regions to test

To obtain the highest possible resolution *i.e.* base pairs, our method virtually tests all the sets of genes that are located in all possible windows of all possible widths. Obviously, testing all window positions and widths is inefficient and yields a lot of redundancy in the computations. For example, when shifting the window by one nucleotide, the resulting window will often be on the same genes. A simple observation overcomes this problem: one will obtain the same results if, instead of considering nucleotides, we consider genes ordered on the

chromosome. Still, the number of computations to perform can be very large as there are $\frac{n(n-1)}{2}$ windows (sets of adjacent genes) on a chromosome with n genes (see figure 1).

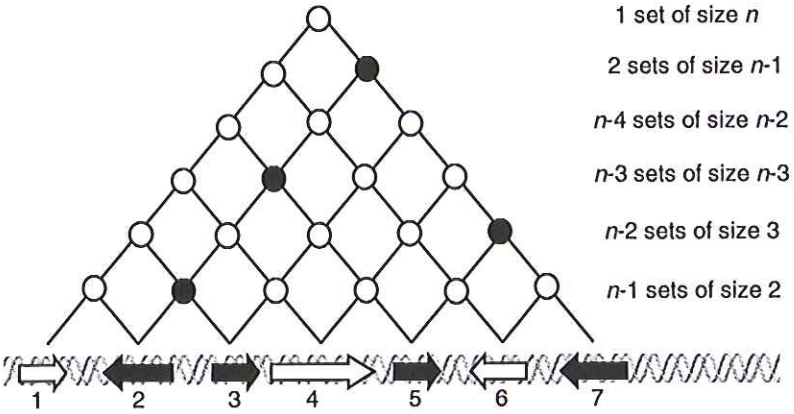

Figure 1: sets of genes that are adjacent on the chromosome. Genes are ordered on the chromosome by their start position (in base pairs). Each pair of genes defines an interval *i.e.* a set of adjacent genes.

The other and more important problem we face is that, with this setup, we will obtain a lot of redundancy in terms of overlapping enriched regions. Let us take for example a region R_1 of 100 genes containing 90 genes of interest. This is very unlikely (*i.e.* significant) and will be reported as an enriched region. If we now consider a slightly larger region R_2 of 101 genes that includes R_1 and that also contains 90 genes of interest, then R_2 will also be reported as enriched. This is statistically true, but reporting R_2 is redundant with R_1 because it is merely the same region to which we add one gene **not** of interest. Hence, R_2 should not be part of the results.

To avoid redundancy and identify the *pertinent regions* to consider, we have adapted the pertinence definition proposed in (Barriot, Sherman and Dutour 2007) to our particular context: a region is considered pertinent if

Rule 1: it contains at least 2 genes of interest,

Rule 2: there is no smaller region containing the same genes of interest, and

Rule 3: there is no bigger region with more genes of interest and the same genes not of interest.

These rules are illustrated in figure 1 with the genes of interest (genes of rank 2, 3, 5 and 7 on the chromosome) and the pertinent regions ([2,3], [2,5], [2,7] and [5,7]) represented by black-filled nodes in the implicit lattice. These rules already reduce the number of test to perform from $n.(n\text{-}1)/2$ (number of sets of adjacent genes) to at most $q(q\text{-}1)/2 = O(q^2)$ (number of regions that start and end with a gene of interest), with q the number of genes of interest.

Unfortunately in practice, those 3 rules do not remove as much redundancy as it might be desirable. Figure 2 illustrates a concrete example of this problem (biological meaning addressed in the discussion section). In this figure, the whole q arm of chromosome 21 is enriched and the largest region has the best *p-value*. As the whole q arm is enriched, one should be tempted to report only this region. However, there might be smaller regions that are worth considering. Indeed, while the *p-value* allows to assess the statistical significance of the enrichment, the percentage of genes of interest in a region provides a raw enrichment measure which is quite informative, also used in (Al-Shahrour, Diaz-Uriarte and Dopazo 2004). In figure 2, we see that the q arm has an enrichment of approximately 20% in genes of interest, and there is also a smaller region that has almost 50% enrichment, thus it might be interesting to consider this smaller more specific region for investigation even if it is statistically less significant. This is because the percentage of enrichment and the *p-value* are biased towards opposite directions: informally, small regions tend to have higher percentages while large regions tend to have better *p-values* (effect size). In other words, for a given percentage, a larger region will have a smaller *p-value* (observing 2/4 is more probable than observing 150/300). To filter and obtain both regions with best *p-values* and best percentage of enrichment, we derive the following rules:

Rule 4: a region included in a larger region with a better *p-value* is pertinent only when it has a higher percentage of genes of interest.

And the converse:

Rule 5: a region is pertinent if it does not include a smaller region with a better *p-value* (if a larger region has a worse *p-value*, then this implies that it includes a smaller percentage of genes of interest).

Finally, given q the number of genes of interest, a region spanning over t genes is expected to contain a certain number of genes of interest ($q / g * t$ with g the total number of genes). In other words, we expect one gene of interest every g/q genes. Based on this observation, we can derive the following rule:

Rule 6: a region is pertinent if it does not contain any region made of g/q adjacent genes that are not of interest.

This last rule allows us to further reduce the search space: before considering all pairs of genes of interest on a chromosome (set of adjacent genes satisfying Rules 1 and 2), the chromosome is split into smaller regions that satisfy Rule 6.

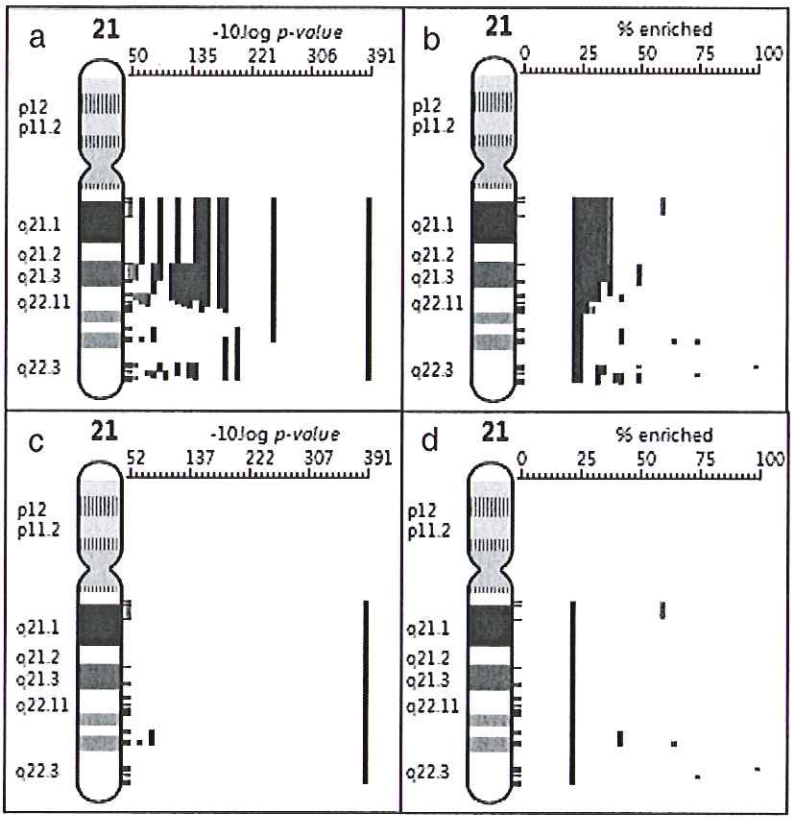

Figure 2: Filtering redundant chromosome regions significantly enriched in genes differentially expressed in tissues of Down syndrome patients on chromosome 21: a) regions are displayed from left to right by increasing *p-value* significance (decreasing p-values) and are plotted as -10.log *p-value*; b) the same regions plotted by the percentage of genes of interest; c) enriched regions are filtered for redundancy and plotted by *p-value*: see rules 4 and 5 in text; d) the same regions plotted by the percentage of enrichment.

Results and discussion

Implementation of the PGE algorithm as a Web interface

The PGE method was implemented as a Perl script that is publicly accessible as a web interface (http://homes.esat.kuleuven.be/~bioiuser/pge/). Currently, users can choose to submit lists of Affymetrix probeset ids or Ensembl Ids. Probeset ids are mapped either to gene symbols or Ensembl ids by using the Affymetrix human genome array plate set annotations file provided on the Affymetrix Web site. As users might be specifically interested to check the enrichment in a specific chromosome, they have the possibility to only calculate enrichment in the chromosome of interest. To build the empirical minimum *p-values* cumulative distribution function for *p-values* significance assessment, we perform 500 simulations with random query sets of the same size as proposed in (Barriot, Poix, Groppi, Barre, Goffard, Sherman, Dutour and de Daruvar 2004). Because of the computational cost of these simulations, this correction method is available only for gene lists of up to 500

genes; otherwise the False Discovery Rate (Benjamini and Hochberg 1995) is applied. The threshold for adjusted *p-value* significance is set to 0.05.The tool visualizes the enriched chromosomal regions along the chromosomes, based on *p-value* or percentage of enrichment. A mouse-over pop-up window gives further information on the Ensembl Ids or gene symbols that are present in the enriched chromosomal region.

Validation and application of PGE on published gene lists

We applied the PGE analysis on published gene lists from microarray gene expression studies in order to validate the algorithm. A first test was performed on a list of 407 genes obtained by comparing genetic subtypes of B-cell chronic lymphocytic leukemia (CLL) (Haslinger, Schweifer, Stilgenbauer, Dohner, Lichter, Kraut, Stratowa and Abseher 2004). In B-CLL, the most frequent losses of genomic material are deletions of chromosome bands 13q14, 11q22-23, 17p13 and 6q21, and the most frequent gains affect 12q13, 8q24 and 3q26. In this study, the authors identified genes that are differentially expressed based on microarray gene expression data in different B-CLL subgroups (considering following genetic parameters: 17p13, 11q22-q23, 13q14, and 6q21 deletion, as well as trisomy 12q13) and they visually noticed that a large number of the differentially expressed genes mapped in the chromosomal regions affected by the respective genomic losses or gains. To confirm this correlation, we submitted the set of differentially expressed genes to our PGE tool which reported significantly enriched regions in agreement with the most frequent genomic alterations (see table 1 and figure 3).

Using the PGE algorithm we also noticed a high degree of correlation between the chromosomal localization of differentially expressed genes and the respective genomic aberrations. We found enrichment of genes on 17p13, entire chromosome 12, 11q23, 6q14.3-q23.2 and 6p21.32-p22.2.

For chromosome 6, we find gene enrichment for the region 6q14.3-q23.2 which includes the frequently deleted chromosomal band 6q21, and enrichment in the region 6p21.32-p22.2. From the figure, it appears that some genes cluster on band q21, but this enrichment is not statistically significant which means that such a cluster could arise by chance. The authors suggest that genes located outside of the gained or lost regions might be downstream effectors of the genes directly affected by the loss or gain and may contribute to the disease phenotype. The region 6p21-p22 contains genes that are members of the *HIST1* major histone gene locus. This HIST cluster enrichment might be due to the fact that Affymetrix probeset-ids for the different HIST genes are not specific enough, or might only indicate that when HIST genes are expressed, the whole cluster is transcribed.

Chr.	Band(s)	Coordinates	Genes of interest / Genes in the region
6	p21.32 - 22.2	26,163,912 - 33,851,518	13/58
6	p22.1	27,208,799 - 27,941,634	7/15
6	p22.1	27,883,200 - 27,941,634	5/7
6	q14.3 - 23.2	86,216,527 - 132,690,949	12/77
11	q14.1 – 24.3	77,603,590 - 127,897,218	18/124
11	q23.1 – 23.3	111,117,019 - 117,775,136	8/28
12	p13.31 – 24.33	7,233,850 - 131,915,071	64/408
12	p11.21 – q13.11	31,117,786 - 44,641,909	5/10
12	q23.3	104,025,639 - 106,630,469	4/5
12	q24.31	120,230,432 - 121,194,727	4/6
17	p13.1 - 13.3	594,403 - 8,006,662	19/90
17	p13.1	7,084,456 - 8,006,662	11/25
17	p13.2	3,746,634 - 4,742,127	5/11

Table 1: Chromosome regions significantly enriched in genes differentially expressed in subtypes of B-CLL.

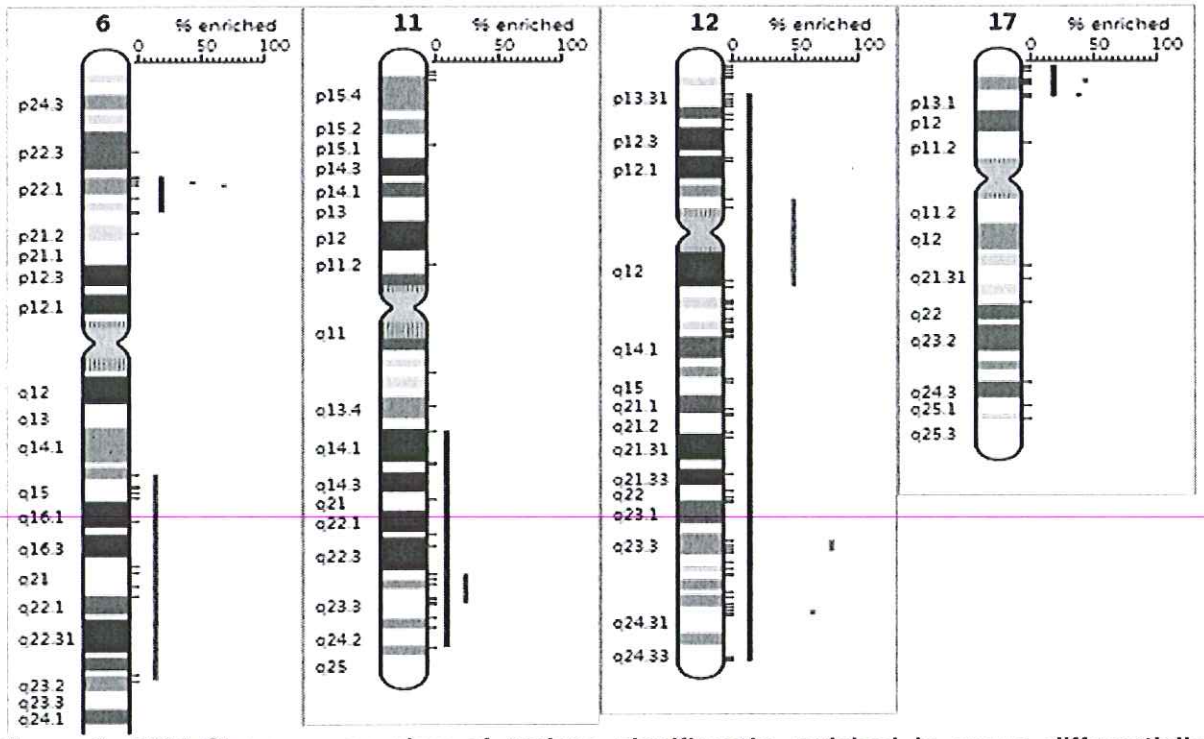

Figure 3: PGE Chromosome view of regions significantly enriched in genes differentially expressed in subtypes of B-CLL (Table 1).

With our tool, we see that the loss of 13q14 do not lead to clusters of genes differentially expressed in that region. By correcting the *p-values* with the False Discovery Rate (Benjamini and Hochberg 1995), this appeared to be significantly enriched (data not shown). This highlights the importance of the multiple testing adjustment method applied. In this case,

the minimum *p-values* distribution function shows that the number of differentially expressed genes observed on 13q14 could have occurred by chance by selecting 407 genes at random in the human genome.

For a second test, we mapped the genes that are higher expressed in neuroblastoma tumors with amplification of the proto-oncogene *MYCN*, located on cytoband 2p24, compared to normal neuroblasts (De Preter, Vandesompele, Heimann, Yigit, Beckman, Schramm, Eggert, Stallings, Benoit, Renard et al. 2006). A region with high significance for overrepresentation is found on 2p. Within this region a smaller significant region with a higher enrichment percentage is found at band 2p24 (from 5.8 Mb to 24.1 Mb), containing the *MYCN*, *DDX1*, *NAG* and *VSNL1* genes (Figure 4). *DDX1* and *NAG* are known to be frequently co-amplified and overexpressed in *MYCN* amplified neuroblastomas. PGE on the same dataset also shows an overrepresentation of genes on 17q which is in concordance with the finding that neuroblastoma tumors with MYCN amplification almost always present with gain of the long arm of chromosome 17.

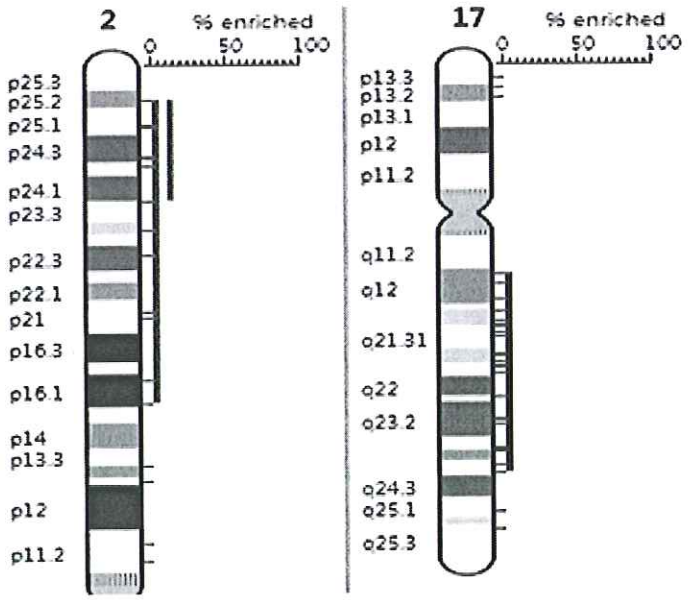

Figure 4: Regions significantly enriched in genes differentially expressed in neuroblastoma tumors.

As a third example, we mapped the genes that are higher expressed in tissues from Down syndrome patients compared to normal samples (Mao, Zielke, Zielke and Pevsner 2003) and found a significant overrepresentation of genes on chromosome 21 (Figure 2). A smaller region with very high significance emerged that contains the *DSCR2* gene that is located in the minimal region for Down syndrome and is a candidate to be involved in the dosage effect of trisomy 21.

Conclusions

In this paper, we present a new efficient method for the high resolution identification of chromosomal regions that are overrepresented in custom gene lists together with its application to the human genome. In contrast to many other methods, PGE does not require any user analysis parameter, is user-friendly and performs rapid calculations (few seconds for gene lists of 100 to 500 genes). The unique approach of this algorithm allows to exhaustively evaluate the overrepresentation rate at all resolution levels simultaneously *i.e.* from pairs of genes to entire chromosomes. The simulations performed for assessing the significance of enriched regions are essential because they allow discriminating which regions are truly enriched from those that could occur by chance. Application of the PGE analysis on published differentially expressed gene lists showed an overrepresentation of aberrant chromosomal regions, demonstrating the validity of the tool. Moreover, within these regions, smaller regions with significant overrepresentation (lower significance but higher enrichment) might highlight specific genes that are of interest in the aberrant regions, and which deserve further study.

In the near future, we will extend the tool with additional identifier types (RefSeq, HGNC, ...) and species (mouse, fly, worm, yeast, ...). The software is generic in the sense that only a reference dataset file mapping identifiers to chromosome locations is needed to propose other identifiers or species.

We will also adapt and implement our method in the context of circular genomes. Actually, very little modifications will be needed: either Rule 6 allows removing at least one region that cannot be enriched and we are left with a linear chromosome, or we have to consider that a pair of (query) genes defines two regions. In the latter case, the worst case time complexity of our method is still in $O(q^2)$ with q the number of genes of interest.

In this article, we focused on gene expression correlated with genomic alteration. This method and tool will also be valuable to test other hypotheses such as tissue-specific chromosomal region accessibility and expression with sets of genes corresponding to EST expressed in different tissues, and genes participating in the same pathways or biological processes.

Author's contributions

RB designed and implemented the method and web interface, and drafted the manuscript. KDP participated in the study design, performed analysis on published gene lists and drafted the manuscript. JV participated in the study design and the interpretation of the results. FS and YM were the final editors of the manuscript. All authors read and approved the final manuscript.

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