Loss of heterozygosity: what is it good for?

Georgina L. Ryland1,2, Maria A. Doyle3, David Goode4, Samantha E. Boyle1, David Y.H. Choong1, Simone M. Rowley1, Jason Li5, Australian Ovarian Cancer Study Group6, David DL Bowtell7, Richard W. Tothill8, Ian G. Campbell1,5,9 and Kylie L. Gorringe1,5,9*

Abstract

Background: Loss of heterozygosity (LOH) is a common genetic event in cancer development, and is known to be involved in the somatic loss of wild-type alleles in many inherited cancer syndromes. The wider involvement of LOH in cancer is assumed to relate to unmasking a somatically mutated tumour suppressor gene through loss of the wild type allele.

Methods: We analysed 86 ovarian carcinomas for mutations in 980 genes selected on the basis of their location in common regions of LOH.

Results: We identified 36 significantly mutated genes, but these could only partly account for the quanta of LOH in the samples. Using our own and TCGA data we then evaluated five possible models to explain the selection for non-random accumulation of LOH in ovarian cancer genomes: 1. Classic two-hit hypothesis: high frequency biallelic genetic inactivation of tumour suppressor genes. 2. Epigenetic two-hit hypothesis: biallelic inactivation through methylation and LOH. 3. Multiple alternate-gene biallelic inactivation: low frequency gene disruption. 4. Haplo-insufficiency: Single copy gene disruption. 5. Modified two-hit hypothesis: reduction to homozygosity of low penetrance germline predisposition alleles. We determined that while high-frequency biallelic gene inactivation under model 1 is rare, regions of LOH (particularly copy-number neutral LOH) are enriched for deleterious mutations and increased promoter methylation, while copy-number loss LOH regions are likely to contain under-expressed genes suggestive of haploinsufficiency. Reduction to homozygosity of cancer predisposition SNPs may also play a minor role.

Conclusion: It is likely that selection for regions of LOH depends on its effect on multiple genes. Selection for copy number neutral LOH may better fit the classic two-hit model whereas selection for copy number loss may be attributed to its effect on multi-gene haploinsufficiency. LOH mapping alone is unlikely to be successful in identifying novel tumour suppressor genes; a combined approach may be more effective.

Keywords: Tumour suppressor gene, SNP, Ovarian cancer, Mutation, Haploinsufficiency

Background

Cancer cells undergo multiple genetic and epigenetic hits in the development of tumorigenic phenotypes, including somatic point mutations, increases in copy number, gene deletions, gene rearrangements, translocations and promoter hypermethylation [1]. These random events are selected for due to their effect on oncogenes, where the aberration activates the gene to promote tumorigenesis (e.g. KRAS, MYC), and on tumour suppressor genes (TSG), where the genetic or epigenetic aberrations is inactivating (e.g. TP53, PTEN), since the normal function of these genes is to restrict tumorigenic potential.

Loss of heterozygosity (LOH) is a common genetic event in many cancer types, so-called because of the early observations of a change in polymorphic markers from a heterozygous state in the germline to an apparently homozygous state in the tumour DNA [2]. LOH is a general term that encompasses both LOH with copy number losses (CNL-LOH) and copy number neutral LOH (CNN-LOH). In CNL-LOH all or part of a chromosome is deleted. CNN-LOH originates either
through a homologous recombination event ("gene conversion"), or because the retained chromosome was duplicated either before or after the LOH event. LOH is strongly associated with loss of the wild-type allele in individuals with an inherited cancer predisposition syndrome and carry a germline mutation in genes such as RB1 in retinoblastoma or BRCA1 in breast and ovarian cancer [2, 3]. This "second hit" hypothesis was initially proposed by Knudson based on his observations of the incidence of familial retinoblastoma [4] and has been widely accepted as a mechanism for the complete inactivation of tumour suppressor genes, both in a germline context and the sporadic cancer context where the first hit is a somatic event, such as mutation of TP53. As a consequence, mapping of common regions of minimal LOH has historically been a popular strategy to pursue the identification of novel TSGs without the need for segregation data from large cancer families. However, such analyses have been generally been unsuccessful leading to speculation that the approach is technically and conceptually flawed [5], and even to whether there is any selective advantage to LOH events. Nonetheless, we previously used SNP mapping arrays to analyse LOH in ovarian carcinomas of diverse histological subtypes, with the rationale that the newer methodology would at least overcome some of the previous technical issues with LOH analyses [6]. We mapped a number of minimal regions of LOH containing tumour suppressor gene candidates, including regions of homozygous deletion encompassing genes such as MAP2K4 [7]. Advances in massively parallel sequencing has enabled the current study where we report targeted sequencing of 980 candidate tumour suppressor genes in 86 ovarian carcinomas, most of which have matched SNP array data enabling the assessment of the importance of LOH in the selection for somatic mutations in ovarian cancer. We evaluated a number of different histological subtypes, since these have different etiologies and causative genes.

### Methods

#### Ethics statement

Accrual and use of patient material for this study was approved by the following Human Research Ethics Committees: Peter MacCallum Cancer Centre Human Research Ethics Committee, Southampton Hospital Human Research Ethics Committee, University of Melbourne Human Research Ethics Committee, Queensland Institute of Medical Research Human Research Ethics Committee, Westmead Hospital Human Research Ethics Committee. All individuals gave written informed consent for the use of their tissue in research. This project was approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (Approval # 09/29).

#### Ovarian tumour cohort

A tumour cohort (n = 86) comprising a variety of histological subtypes including serous (n = 45), endometrioid (n = 28), mucinous (n = 7) and clear cell (n = 6) were obtained through the Australia Ovarian Cancer Study, the Peter MacCallum Cancer Centre Tissue Bank, or from patients presenting to hospitals in the south of England [8]. The majority of tumour DNA samples were needle microdissected to ensure greater than 70 % cancer epithelial cell component; other samples were processed from tissue where the reference haematoxylin and eosin stained section showed >70 % tumour epithelial cells. Matching peripheral blood samples were also collected from patients at time of tumour collection and used as a source of germline DNA for somatic mutation detection. Details of the cohort are listed in Additional file 1: Table S1.

#### Library preparation, target enrichment and sequencing

Library preparation was performed as previously described [9] following the Illumina genomic DNA library preparation protocol (Illumina, San Diego, CA) using an input of 200 ng of tumour or matched normal lymphocyte DNA. Seven custom multiplexing adapters compatible with Illumina single-end sequencing were used and indexed DNA samples were pooled equally prior to PCR enrichment. A boutique exon capture (SureSelect, Agilent Technologies, Santa Clara, CA) was used to enrich for coding exons of candidate tumour suppressor genes (n = 980, Additional file 1: Tables S2 and S3) and known cancer genes (TP53, BRCA1, BRCA2) according to the recommended protocol. Capture probes were designed using default parameters in eArray (Agilent Technologies).

Sequencing of target-enriched DNA libraries were performed using an Illumina GAIIx, generating 75 bp single-end sequence reads. Image analysis and base calling was performed using the Genome Analyser Pipeline v1.5-1.7. Sequence reads were aligned to the human reference genome (GRCh37/hg19 assembly) using BWA [10] and any remaining unmapped reads aligned with Novoalign [11]. The mean coverage for bases within target regions was 70-fold and 92 % had at least 10-fold coverage. This was followed by local realignment with GATK [12]. Point mutations and insertions/deletions (indels) were identified using GATK and Dindel [13] respectively, and annotated according to Ensembl release 66. Sequence variants were called as somatic alterations only when (i) the variant was not called in the matched normal sample or identified as a germline alteration in another tumour/normal pair (ii) the variant was not seen in >2 independent reads in the matched normal sample following manual inspection of sequence reads using the Integrated Genomics Viewer [14] (iii) the variant was identified in bi-directional sequence reads.
tp53 where a high proportion of – (2015) 8:45 or et al. BMC Medical Genomics tp53 and somatic mutations brca1 correlation of tp53 allele had to have a value of <0.5 cdkn2a brca2 LOH genes. In addition, the known ovarian tp53 brca1 mutations in 56 cases and two mutations in tp53 and brca1 were included and selection of – . who constitute the TCGA research network can be found . Information about TCGA and the investigators and institutions based upon data generated by The Cancer Genome Atlas present. The results published here are in whole or part impaction frequency of 13 % (72/561). In addition, among the 53 cases with tp53 or brca1 somatic mutations where SNP data was available, 50 (94 %) showed LOH of the wild-type allele. This was is sharp contrast with the other candidate genes, where only 181/520 showed LOH of the wild-type allele (35 %); in particular, there was no significant difference in LOH of the wild-type allele between non-synonymous mutations (134/381 with LOH, 35.2 %) and synonymous mutations (47/139 with LOH, 33.8 %). The overall frequency of non-synonymous compared to synonymous mutations was 73 % (411/561) for the candidate TSGs, but 100 % of mutations in known cancer genes were non-synonymous (60/60). This difference in ratio suggests that the majority of mutations in candidate TSGs from LOH regions are likely to be passenger events, since this rate might be expected without any strong positive selection [16]. The lack of difference in LOH between synonymous and non-synonymous also implies that there is limited selection for homozygosity for the majority of gene mutations.

Significance analysis of recurrently mutated gene candidates

Within the list of mutated genes, we applied a number of filters to assess whether any genes could function as tumor suppressors under either a one-hit or two-hit mechanism. Firstly, significantly mutated genes were identified using the MuSiC algorithm [17], which determines the significance of the observed mutation rate of each gene based on the background mutation rate in the sample cohort. Three known ovarian cancer genes (TP53,
PTEN and CDKN2A) were identified by all three tests (convolution, likelihood ratio and Fisher’s combined p-value tests) with a false discovery rate (FDR) of less than 0.10. At this FDR the genes DNAH9, LINGO1, MEF2C, SAMD11, STARD5, ZNF4 and ZNF287 were also identified, although each was supported only by the likelihood ratio test.

Secondly, the 125 genes with recurrent mutations were assessed for the proportion of cases with biallelic mutation, including by homozygous deletion from SNP array data. For genes with mutations in three or more cases, the proportion of biallelic mutations was greater than 80% of mutated samples for eight genes (AL355987.1, CASK, CDKN2A, MAP2K4, NF1, PTEN, RB1 and TP53) and between 60-80% for seven genes (FANCA, GRAMD4, GPR98, IL16, MYOCD, SYNE1 and TEX15). Biallelic mutations were detected in 2/2 mutated samples in an additional nine genes (SKG223, APOOL, BRCA1, CDH8, DACH2, EPHX2, FARP1, PNMA3 and RAI1).

Finally, genes recurrently targeted by inactivating mutations were identified. Mutations with overtly deleterious consequences were considered for this analysis, including nonsense and essential splice site mutations, frameshift indels and gene deletions. Although missense amino acid changes and in-frame indels can also negatively impact gene function, interpreting these mutations in the absence of functional validation is challenging. Sixteen genes were identified where more than half of their mutations would be considered clearly deleterious, including seven known ovarian cancer genes (PTEN, CDKN2A, MAP2K4, PIK3R1, RB1, FANCA and BRCA1). These three analyses identified 36 genes as possible tumour suppressors (Table 1), and it was notable that seven well characterised tumour suppressors were identified by at least two of the three methods (BRCA1, TP53, RB1, PTEN, CDKN2A, FANCA, and MAP2K4), although others were only identified by one method (NF1, PIK3R1). In contrast, 22 of the 27 (81%) novel/less well characterized genes were identified by only 1 method, indicating that regions of LOH are not strongly enriching for novel genes with classic tumor suppressor gene characteristics.

Loss of heterozygosity – what is it good for?

From the data above it appears that we did not identify dominant, very frequently mutated novel genes where selection for a classic two-hit tumour suppressor gene was apparent. So what, if anything, is the LOH for? We considered five possibilities (Fig. 1) and assessed each in turn.

1. Classic two-hit hypothesis: high frequency biallelic genetic inactivation of TSG

2. Epigenetic two-hit hypothesis: biallelic inactivation through methylation and LOH

3. Multiple alternate gene biallelic inactivation: low frequency gene disruption

4. Haplo-insufficiency: Single copy gene disruption

5. Modified two-hit hypothesis: reduction to homozygosity of predisposition alleles

This mechanism is demonstrably true for many known tumour suppressor genes, with TP53 being a clear example of a gene functioning as a classical TSG in ovarian cancer [18, 19]. However, from our data and large published studies such as TCGA, it is clear that novel genes with a high frequency of biallelic mutations are exceedingly rare and can not explain the bulk of the observed LOH. For example, 8p undergoes LOH in >40% of ovarian carcinomas, but no gene in this region is mutated at frequency higher than 3% in our or any other study, although homozygous deletion can target, for example, CSMD1 in 11% of cases [20]. It remains a possibility, however, that genes not represented on our targeted or exome sequencing platforms could still be the target of such LOH, for example long non-coding RNAs.

2. Epigenetic two-hit hypothesis: high frequency biallelic inactivation through methylation and LOH

Somatic gene mutation is not the only mechanism of biallelic inactivation. Some TSGs can be inactivated through a combination of LOH and enhancer hypermethylation, for example MLH1. This methylation can be acquired somatically or may be a consequence of imprinting. We assessed this possibility using TCGA ovarian cancer methylation data. Globally, we observed that there was no enrichment for methylation in regions of LOH – in samples with LOH at a locus, on average 12.7% of genes were strongly methylated (probe value of >0.75), whereas 13.65% of genes were strongly methylated when there was no LOH, (Fig. 2a). CNL-LOH was less likely to have strongly methylated genes than CNN-LOH (12.3% vs 12.9%, p < 0.0001, Chi-squared test). However, when we analysed the X chromosome separately, we found that samples with any LOH were more likely to have low methylation levels (45.3% of genes had a probe value of <0.25, compared to 35.5% in samples without LOH, p < 0.0001 Chi-squared test).

Detection of methylation is challenging from both technical and biological perspectives. Tumour and cell
| Gene     | Location | Description                                      | Recessive TSG* | Predominant subtypes | TCGA mutated* | Detected by       |
|----------|----------|--------------------------------------------------|----------------|-----------------------|---------------|-------------------|
| ANKRD32  | 5q15     | ankyrin repeat domain 32                        | HG S/E + LG E + CC | 9                     | Deleterious    |
| APOUL    | Xq21.1   | apolipoprotein O-like                           | HG S/E         | 0                     | Biallelic      |
| BRCA1    | 17q21.31 | breast cancer 1, early onset                     | HG S/E         | 12                    | Biallelic, Deleterious |
| C9orf17  | 9q34.3   | chromosome 9 open reading frame 172             | HG S/E         | 5                     | Biallelic      |
| CACNA1B  | 9q34     | calcium channel, voltage-dependent, N type, alpha 1B subunit | LG E + CC   | 7                     | Deleterious    |
| CASK     | Xp11.4   | calcium/calmodulin-dependent serine protein kinase | HG S/E       | 2                     | Biallelic      |
| CDH8     | 16q22.1  | cadherin 8, type 2                              | HG S/E         | 9                     | Biallelic      |
| CDKN2A   | 9p21     | cyclin-dependent kinase inhibitor 2A             | Y              | Muc                   | 8              | MuSiC, Biallelic, Deleterious |
| CYLC1    | Xq21.1   | cyclin, basic protein of sperm head cytoskeleton 1 | HG S/E       | 4                     | Deleterious    |
| DACH2    | Xq21.3   | dachshund homolog 2 (Drosophila)                | HG S/E         | 3                     | Biallelic      |
| DNAH9    | 17p12    | dynein, axonemal, heavy chain 9                 | LG E           | 16                    | MuSiC         |
| EPHX2    | 8p21     | epoxide hydrolase 2, cytoplasmic                | HG S/E         | 12                    | Biallelic      |
| FANCA    | 16q24.3  | Fanconi anemia, complementation group A         | Y              | HG S/E                | 12             | Biallelic, Deleterious |
| FARP1    | 13q32.2  | FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 | HG S/E       | 3                     | Biallelic      |
| GPRR8    | 5q13     | G protein-coupled receptor 98                    | HG S/E         | 17                    | Biallelic      |
| GRAMD4   | 22q13.31 | GRAM domain containing 4                        | HG S/E         | 18                    | Biallelic, Deleterious |
| IL16     | 15q26.3  | interleukin 16                                  | HG S/E         | 4                     | Biallelic, Deleterious |
| LINGO1   | 15q24.3  | leucine rich repeat and Ig domain containing 1   | HG S/E         | 4                     | MuSiC         |
| MAP2K4   | 17p12    | mitogen-activated protein kinase kinase 4       | Y              | HG S/E                | 12             | Biallelic, Deleterious |
| MEF2C    | 5q14.3   | myocyte enhancer factor 2C                      | HG S/E         | 8                     | MuSiC         |
| MYOCD    | 17p11.2  | myocardin                                       | HG S/E         | 7                     | Biallelic, Deleterious |
| NF1      | 17q11.2  | neurofibromin 1                                 | Y              | HG S/E                | 37             | Biallelic         |
| PKR1     | 5q13.1   | phosphoinositide-3-kinase, regulatory subunit 1 (alpha) | Y              | LG E                  | 6              | Deleterious       |
| PNMA3    | Xq28     | paraneoplastic Ma antigen 3                     | HG S/E         | 2                     | Biallelic      |
| PTEN     | 10q23    | phosphatase and tensin homolog                   | Y              | LG E                  | 25             | MuSiC, Biallelic, Deleterious |
| RAF1     | 17p11.2  | retinoic acid induced 1                         | HG S/E         | 4                     | Biallelic      |
| RB1      | 13q14.2  | retinoblastoma 1                                | Y              | HG S/E                | 32             | Biallelic, Deleterious |
| Rps6ka6  | Xq21.1   | ribosomal protein 56 kinase, 90 kDa, polypeptide 6 | HG S/E + LG E | 0                     | Deleterious    |
| SAMD11   | 1p36.33  | sterile alpha motif domain containing 11        | HG S/E         | 7                     | MuSiC, Deleterious |
| SKG223   | 8p23.1   | Sugen kinase 223                                | HG S/E + Muc   | 7                     | Biallelic      |
| STARD5   | 15q26    | StAR-related lipid transfer (START) domain containing 5 | HG S/E       | 1                     | MuSiC         |
| SYNE1    | 6q25     | spectrin repeat containing, nuclear envelope 1   | HG S/E         | 14                    | Biallelic      |
type heterogeneity may influence the degree of methylation detected, so we also took an alternative approach where we used the methylation array data to test whether there were genes that were more strongly methylated in samples with LOH compared to samples without LOH. Using a multiple testing correction p-value threshold of $2.2 \times 10^{-6}$, there were 1584/22374 (7 %) methylation probes that were significantly differentially methylated. Interestingly, 28 % of these significant probes were located on the X chromosome and indeed 51 % of all probes on the X chromosome were significantly differentially methylated, with lower average levels of methylation in samples with LOH compared to samples without LOH. On the autosomes, the outcome was reversed: 50.3 % of the statistically significant probes had a fold-change difference in mean methylation of $>1.5$, while only 1.1 % had a fold-change difference of $<0.75$ (Fig. 2b). Thus, for the X chromosome it appears there is selection for retaining the active copy, perhaps because loss of this copy would be cell lethal as an effective homozygous inactivation of the chromosome. In contrast, for the autosomes there appears to be selection for increased methylation by LOH.

We also evaluated whether there was any difference by the type of LOH. For those genes occurring in a region of LOH with at least 20 % frequency, we determined whether a probe was in a CNL-LOH enriched locus (>66 % of samples with LOH also had CN loss) or a CNN-LOH enriched locus (>66 % of samples with LOH were CNN). Of the CNL-enriched probes, 11.3 % were significantly differentially methylated, compared to 21.7 % of CNN-enriched probes ($p < 0.0001$, Chi-squared test, Fig. 2c). This data would support a model whereby differential methylation is more commonly selected for in regions of CNN-LOH than CNL-LOH.

### 3. Multiple alternate gene biallelic inactivation: low frequency gene disruption

Another possibility is that particular loci harbour multiple TSGs but individual tumours only require one to be inactivated and the gene targeted can differ from tumour to tumour. If this is the case then locating the TSGs by mapping overlapping regions of LOH would incorrectly flag the interval between two TSG as the likely location of the TSG – in effect then the peak LOH regions may not be the most likely places to find the targeted gene(s). To evaluate this possibility, we used TCGA data to see whether regions of LOH were enriched for somatic mutations on a sample-by-sample basis. Cases with both somatic exome and SNP array data were used ($n = 266$). There were 13,148 coding somatic mutations, of which 29.7 % were located within a region of LOH in the sample where it was observed. The average overlap of all the genes assayed with regions of LOH per sample was 35.5 %.

### Table 1 Selected mutated genes in candidate TSG screen (Continued)

| TSG    | Chromosome and Protein          | HG S/E | Muticlass  |
|--------|---------------------------------|--------|------------|
| ZNF287 | 17p11.2 zinc finger protein 287 | HG S/E | 4          |
| ZNF287 | 17p11.2 zinc finger protein 287 | HG S/E | 4          |

LG E, low-grade endometrioid; HG S/E, high-grade serous/endometrioid; Muc, mucinous; CC, clear cell

*known recessive tumour suppressor gene according to the Cancer Gene Census (32)

*number of high-grade serous TCGA samples with somatic point mutations and indels including large homozygous deletions. Mutation data for 316 TCGA samples (33) was accessed through the cbio Cancer Genomic Portal (34)
expected (10.3 % vs 8.7 %, p = 0.05, Binomial test). When TP53 was included, both total mutations (9.2 %) and deleterious mutations (11.4 %) in CNN-LOH regions were increased. Silent mutations were the most likely to be underrepresented in CNL-LOH regions (17.2 % vs 26.8 %).

It is possible, therefore, that mutations are seen less often in CNL-LOH regions simply as a consequence of decreased DNA dosage. The enrichment of deleterious mutations in CNN-LOH regions, however, suggests the presence of positive selection for mutations in TSGs.
4. Haplo-insufficiency: Single copy gene disruption

We and others have shown that loss of a single gene copy can reduce gene expression [6, 21]. A recent study showed that regions of copy number loss are enriched for tumour-suppressor genes [22], but that each gene might have a limited effect on its own. Chromosome complementation studies, where all or part of a chromosome is introduced into cell lines with LOH of that chromosome via microcell-mediated monochromosome transfer, have frequently been able to show reduction in tumorigenicity of the cell line thus complemented [23, 24], but have only rarely been able to implicate a single gene responsible [25, 26]. Thus, haplo-insufficiency of multiple genes, each with a small effect, could contribute to the non-random pattern of LOH observed in ovarian cancer, especially for chromosomal regions that are weighted towards CNL-LOH such as 8p and X, rather than CNN-LOH, such as 17.

We previously observed a correlation between the percentage of genes under-expressed and the percentage of cases with CNL-LOH, as opposed to CNN-LOH, in a region-wise comparison of LOH vs. no LOH [6]. In an analysis of TCGA data, we compared the expression of...
10,925 genes between cases with and without LOH. 3,780 genes (34.6%) were significantly differentially expressed (at a multiple testing p-value threshold of $4.56 \times 10^{-6}$), and all significant genes were under-expressed in samples with LOH compared to samples without LOH. When comparing CNN-LOH to CNL-LOH in genes with at least 20% frequency of LOH, only 1/163 genes (0.6%) at CNN-LOH enriched loci were significantly differentially expressed, compared to 2701/5740 (47%) CNL-LOH enriched genes ($p < 0.0001$, Chi-squared test, Fig. 4). This result supports the idea that chromosomal regions with CNL-LOH may contain genes where loss of a single copy results in reduced gene expression and a selective advantage to the cell. In contrast, chromosomal regions with little copy number loss may contain essential genes for which haplo-insufficiency is cell lethal.

5. Modified two-hit hypothesis: reduction to homozygosity of predisposition alleles

In familial cancer predisposition syndromes, it is common for the remaining wild-type allele to be lost by LOH, for example, *BRCA1* pathogenic variants are usually reduced to homozygosity in breast and ovarian carcinomas [3, 27]. However, common low-penetrance risk alleles could also be targeted by LOH leading to an enhancement of their cancer-promoting role. We assessed this using nine SNP loci identified in the iCOGs study [28, 29] as predisposing to all ovarian cancer types or high-grade serous ovarian cancer. Two of these SNPs were present on the Affymetrix SNP 6.0 array, the remainder were represented by SNPs in linkage disequilibrium ($r^2 > 0.7$, from HapMap [30]). Where possible, up to four linked SNPs were evaluated.

For each SNP, we assessed whether cases were heterozygous in their normal DNA, and what proportion of these with LOH of the region were homozygous for the risk allele in the tumour DNA using TCGA and our own data (n = 364). Interestingly, two SNPs at 10p12 linked to the risk allele rs1232180 were found to be significantly more likely to have lost the non-risk allele than the risk allele (Table 2). Some other SNPs linked to a risk allele also showed significantly non-random loss of the non-risk allele, but the data were not consistent across all SNPs examined at the locus (e.g. 17q21, 3q25 and 9p22). It is not clear whether these discrepancies could be due to technical variation in the SNP calling; alternative methods may be required to assess this possibility. The remainder of SNPs were not significant, however several are uncommon, limiting the power of the analysis. Thus, it is possible that some LOH may be selected for through the phenotypic effect of reduction to homozygosity of predisposition alleles.

**Conclusion**

The broader relevance of LOH in cancer has been debated for some time [5, 31] although many of the criticisms stemmed from technical issues that are being overcome by newer methodologies. Our initial assumption for this study was that we would detect high-frequency mutated genes in the minimal peak regions of LOH we had defined by LOH mapping using these newer methodologies; i.e. a classic two-hit model. However, the biology of LOH does not support this assumption and with large-scale tumour studies it is now possible to explore the many possibilities for the functional significance of this genetic event as summarised in Table 3. We suggest that the non-random patterns of LOH detected in cancer are a result of multiple different mechanisms operating to affect multiple genes, which may differ from tumour to tumour yet collectively play a role in the development of the tumorigenic phenotype. It is worth noting the differences in CNL-LOH versus CNN-LOH, with the latter appearing more relevant for selection of deleterious mutations and methylation, in contrast to global changes in gene expression. Identifying the specific driver genes targeted in a particular cancer remains a challenge given the multiple possible reasons for selection of an LOH event.
### Table 3 Summary of LOH – what is it good for?

| Hypothesis                      | Mechanism                                                                 | Plausibility | Frequency | Impact | LOH type   |
|---------------------------------|---------------------------------------------------------------------------|--------------|-----------|--------|------------|
| Classic two-hit hypothesis      | High frequency biallelic genetic inactivation of TSG via mutation and LOH or homozygous deletion | Strong       | Rare      | High   | More CNN-LOH |
| Modified two-hit hypothesis     | Reduction to homozygosity of predisposition alleles                       | Low          | Rare      | Low    | Unknown    |
| Epigenetic two-hit hypothesis   | Biallelic inactivation through methylation and LOH                        | Moderate     | Unknown   | Moderate| More CNN-LOH |
| Haplo-insufficiency             | Single copy gene disruption through copy number loss                      | Strong       | Common    | Moderate| CNL-LOH    |
| Multi-gene biallelic inactivation| Low frequency gene disruption through all of the above mechanisms         | Strong       | Common    | Unknown| Either      |

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### Table 2 Ovarian cancer GWAS SNPs and LOH

| SNP   | Risk Allele | Locus | het | horn | AA | BB | NC | N | CHI sq | Direction | % LOH | Affy SNP   | Rsquared |
|-------|-------------|-------|-----|------|----|----|----|----|--------|-----------|-------|------------|----------|
| rs1243180 | NA          | 1p12  |     |      |    |    |    |    |        |           |       |            |          |
| rs1243188 | minor/A     | 1p12  | 112 | 183  | 22 | 6  | 41 | 364| 0.0025 | yes       | 0.20  | SNP_A-2024177 | 0.881    |
| rs7098100 | minor/B     | 1p12  | 132 | 181  | 1  | 9  | 41 | 364| 0.0114 | yes       | 0.07  | SNP_A-8636193 | 0.781    |
| rs757210  | NA          | 1q12  |     |      |    |    |    |    |        |           |       |            |          |
| rs11658063 | minor/B    | 1q12  | 23  | 148  | 44 | 44 | 105| 364| 1.0000 | .         | 0.79  | SNP_A-8714923 | 0.704    |
| rs9303542 | NA          | 1q21  |     |      |    |    |    |    |        |           |       |            |          |
| rs4451990 | minor/B     | 1q21  | 20  | 197  | 49 | 61 | 37 | 364| 0.2526 | .         | 0.85  | SNP_A-2282117 | 1        |
| rs12944592 | minor/B    | 1q21  | 24  | 198  | 54 | 60 | 28 | 364| 0.5741 | .         | 0.83  | SNP_A-1836563 | 1        |
| rs12452212 | minor/A    | 1q21  | 19  | 196  | 74 | 53 | 22 | 364| 0.0624 | yes      | 0.87  | SNP_A-2128564 | 1        |
| rs9894812 | minor/A     | 1q21  | 20  | 198  | 70 | 48 | 28 | 364| 0.0428 | yes      | 0.86  | SNP_A-2209606 | 1        |
| rs18170   | NA          | 1p13  |     |      |    |    |    |    |        |           |       |            |          |
| rs34084277 | minor/B    | 1p13  | 80  | 260  | 9  | 8  | 7  | 364| 0.0804 | .         | 0.18  | SNP_A-1788674 | 1        |
| rs2072590 | NA          | 2q31  |     |      |    |    |    |    |        |           |       |            |          |
| rs711830  | minor/B     | 2q31  | 77  | 160  | 8  | 14 | 105| 364| 0.2008 | .         | 0.22  | SNP_A-8652216 | 0.965    |
| rs7651446 | NA          | 3q25  |     |      |    |    |    |    |        |           |       |            |          |
| rs344008  | minor/A     | 3q25  | 58  | 297  | 2  | 3  | 4  | 364| 0.6547 | .         | 0.08  | SNP_A-8543714 | 0.85     |
| rs2292336 | minor/B     | 3q25  | 48  | 299  | 2  | 5  | 10 | 364| 0.2568 | .         | 0.13  | SNP_A-8587822 | 0.85     |
| rs17380639 | minor/A    | 3q25  | 28  | 320  | 11 | 3  | 2  | 364| 0.0325 | yes      | 0.33  | SNP_A-2078455 | 0.85     |
| rs11782652 | minor/A    | 8q21  | 38  | 196  | 8  | 7  | 20 | 364| 0.7963 | .         | 0.28  | SNP_A-8702651 | .        |
| rs10088218 | major/A    | 8q24  | 47  | 280  | 11 | 11 | 15 | 364| 1.0000 | .         | 0.32  | SNP_A-1801410 | .        |
| rs1516974 | major/A     | 8q24  | 45  | 291  | 4  | 15 | 9  | 364| 0.0116 | no       | 0.30  | SNP_A-2088878 | 1        |
| rs3814113 | NA          | 9p22  |     |      |    |    |    |    |        |           |       |            |          |
| rs7032221 | major/B     | 9p22  | 123 | 201  | 6  | 11 | 23 | 364| 0.2253 | yes      | 0.12  | SNP_A-8603886 | 1        |
| rs10738467 | major/B    | 9p22  | 111 | 206  | 5  | 11 | 31 | 364| 0.1336 | yes      | 0.13  | SNP_A-8328297 | 0.892    |
| rs10962668 | major/B    | 9p22  | 103 | 177  | 7  | 22 | 55 | 364| 0.0053 | yes      | 0.22  | SNP_A-4198891 | 0.794    |

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1 SNPs in bold are those named in the GWAS iCOG publication [29]. All others are linked as indicated by the R-squared value of >0.7. If the minor allele is the risk allele named, it is assumed that this will also be the case for the linked SNP.
2 Minor = risk allele is the less frequent allele in the population. A, B = risk allele corresponds to the “A” or “B” allele respectively in the Affymetrix array nomenclature. NA = not on Affymetrix SNP6 array.
3 Het = Number of cases where germline and tumour are heterozygous, horn = cases where germline is heterozygous, AA, BB = germline is heterozygous, tumour is homozygous for A or B respectively, NC = no call in either tumour or germline. N = total number.
4 % LOH is the number of individuals with loss of one allele divided by the total number of heterozygous individuals as measured at that SNP, i.e. not the overall % of LOH that could be determined from all cases using a wider genetic window. This may therefore include regions of extreme allelic imbalance (e.g. likely for 8q24).

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Additional files

**Additional file 1: Table S1.** Cohort information. Table S2. Regions of LOH analysed by targeted sequencing. Table S3. List of the genes in regions of LOH analysed by targeted sequencing. (DOCX 135 kb)

**Additional file 2: Table S4.** Excel file with all synonymous and non-synonymous mutations identified by targeted sequencing. (XLSX 141 kb)

**Abbreviations**

AOCs: Australian Ovarian Cancer Study; LOH: Loss of heterozygosity; CNL-LOH: Copy number loss LOH; CNN-LOH: Copy number neutral LOH; SNP: Single nucleotide polymorphism; TCGA: The Cancer Genome Atlas; TSG: Tumour suppressor gene.

**Competing interests**
The authors declare that they have no competing interests.

**Author contributions**

GLR conducted SNP array, mutation validation and all massively parallel sequencing experiments and analysed sequencing data; MAD and JL performed sequencing alignments and variant calling; DG conducted massively parallel sequencing experiments and analysed sequencing data; RWT contributed to assay design and optimisation; IGC conceived of the study, participated in its design and coordination, and KLG performed sequencing alignments and variant calling; GLR conducted SNP array, mutation validation and all clinical and scientific collaborators are: ACT- R Stuart-Harris; NSW- F Kirsten, J Rutovitz, P Clingen, A Glasgow, A Proietto, G Otton, J Shannon, T Manolitsas, J McNealage, P Rogers, B Susil, E Sumithran, P Parker; VIC- B Brown, R Rome, D Allen, P Grant, S Hyde, R Laurie M Robbie, D Henderson, J Miller, J Pierides, A Achani, TAS- D Purdie, D Whitman, B Ward, D Papadimos, A Brandon, M Cummins, K Hongwood, A Obermair, L Perrin, D Wyld, J Nicklin, SA- M Davy, MK de Hoon, Y-N Cheng, I Connolly, T Chadwick, C Shearman, F Delph, N Howlett, R Bell, LF Ng, R Blum, V Ganz, WA- I Hammond, A McCartney (dec.), C Stewart, Y Leung, M Buck, N Zeigler (XREF). Further information can be found at http://www.aocstudy.org. This work was supported by the National Health and Medical Research Council of Australia (NHMRC ID #628773) and the Emer Casey Foundation.

**Author details**

1. Cancer Genetics Laboratory, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.
2. Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia.
3. Bioinformatics Core Facility, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.
4. Bioinformatics and Cancer Genomics Laboratory, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.
5. S2 Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Victoria, Australia. 6. Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.
6. Cancer Genetics and Genetics Laboratory, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.
7. Molecular Genetics Core Facility, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.
8. Department of Pathology, University of Melbourne, Parkville, Victoria, Australia.

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