Utility of temporal artery biopsy samples for genome-wide analysis of giant cell arteritis.

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ABSTRACT

Giant Cell Arteritis (GCA) is the most common vasculitis affecting the elderly. Archived formalin fixed paraffin embedded (FFPE) temporal artery biopsy (TAB) specimens potentially represent a valuable resource for large-scale genetic analysis of this disease.

FFPE TAB samples were obtained from 12 patients with GCA. Extracted TAB DNA was assessed by real time PCR (RT-PCR) prior to restoration using the Illumina HD FFPE Restore Kit. Paired FFPE-blood samples were genotyped on the Illumina OmniExpress FFPE microarray.

The FFPE samples which passed stringent QC measures had a mean genotyping success of >97%. When compared to their matching peripheral blood DNA, the mean discordant heterozygote and homozygote SNP calls were 0.0028 and 0.0003, respectively, which is within the accepted tolerance of reproducibility.

This work demonstrates that it is possible to successfully obtain high quality microarray-based genotypes FFPE TAB samples and that this data is similar to that obtained from peripheral blood.

Giant Cell Arteritis (GCA) is the most common inflammatory vasculitis affecting elderly people. The incidence of GCA increases progressively after the age of 50 years, and it is more common in women [1]. Prevalence of GCA varies depending on ethnic background, with the highest reported incidence in northern Europe and people of Scandinavian descent[2]. GCA typically affects medium and large arteries of the head and neck. Patients can exhibit a variety of ischemic symptoms including headache, jaw claudication, and transient visual obscurations [3]. Involvement of the ophthalmic artery can cause irreversible visual loss. GCA is also a potentially life threatening disease as it can lead to stroke and aortic dissection.

Currently, there are no specific biochemical markers used to identify this disease, though elevated acute phase proteins, reflected by an increased erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) level in conjunction with a compatible clinical picture suggest the diagnosis. Diagnosis of GCA is confirmed histologically by temporal artery biopsy (TAB). TAB is included in the American College of Rheumatology 1990 criteria for the classification of GCA[11] and remains the current gold standard for diagnosis.

To date, studies investigating the genetic architecture of GCA have been limited[4]. However, several small-scale studies have demonstrated an association with MHC class II genes, most notably *HLA-DRB1*04*[5-6]. Significant associations have also been reported for a number of inflammatory cytokines and other small molecules, including but not limited to *TNFa2*,[7] *IL-10*,[8] *NOS2*,[9] and *VEGF*[10]. A comprehensive genome-wide association study (GWAS) of GCA confirming these associations and potentially identifying additional risk loci has yet to be published.

Retrospective recruitment of a large cohort of GCA patients for genetic analysis is challenging. As the mean age of onset of GCA is over 75 years, the primary reason for this issue is that many of the elderly patients live in residential care and are hence difficult to locate, are deceased, or have major medical co-morbidities, precluding participation. To overcome this difficulty in recruitment, we investigated the potential for extracting high quality DNA directly from archived Formalin-Fixed Paraffin-Embedded (FFPE) TABs. Direct comparisons were made to DNA extracted from whole blood.

METHODS

Patients

Ethics approval for this study was obtained from the relevant committees at the Royal Victorian Eye and Ear Hospital (08/823H) and St Vincent's Hospital (026/09), Melbourne, Australia. All study participants provided written, informed consent. Twelve FFPE TAB samples, stored for between three and five years, were obtained from patients with histologically confirmed GCA. These patients also had peripheral blood samples collected for peripheral leucocyte DNA extraction.

TAB DNA Extraction

Ten 10μm sections of each TAB sample were collected and deparaffinised using xylene. The tissue samples were treated with digestion buffer containing proteinase K (1mg/mL proteinase K, 0.5% Tween 20, 100 mM NaCl, 1 mM EDTA, 50 mM Tris HCl pH 7.5) for 24 hours at 55^{o}C. DNA was isolated from the cell lysates by performing two phase-separation steps, using Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Sigma, St. Louis, MO) followed by Chloroform only. DNA was precipitated with isopropanol using a glycogen carrier.

DNA QC Analysis

Double stranded DNA was quantified using Quant-iT^{TM} Picogreen® (Life Technologies, Carlsbad, CA). The quality of the extracted FFPE TAB DNA was evaluated by real time PCR (RT-PCR) using the Illumina Infinium HD FFPE QC Kit (San Diego, CA). Samples which passed quality control proceeded to DNA restoration using the Illumina FFPE Restore Kit.

Genotyping

All 24 samples were genotyped on Illumina Infinium HD FFPE OmniExpress arrays as per manufacturer's instructions. The raw fluorescence intensity data was normalized and clustered for each sample using Illumina Genome Studio (v. 2011.1). Genotypes were called using the standard Illumina HumanOmniExpress FFPE cluster file.

Genotyping quality control, including gender concordance, missing rates, heterozygosity, duplicated individuals and genotyping concordance between FFPE TAB and blood derived DNA, were assessed using PLINK (v1.07) and R (v 2.15.1). The correlation between RT-PCR results and genotyping missing rate was determined by linear regression.

RESULTS AND DISCUSSION

DNA of sufficient quantity for microarray genotyping was extracted from all 12 FFPE TAB samples. RT-PCR analysis indicated that all FFPE TAB samples met the manufacturer's criteria (a RT-PCR Δ cycle threshold (CT) value of <5) for proceeding to restoration and genotyping.

Using the Illumina OmniExpress FFPE cluster file all peripheral blood DNA samples were successfully genotyped with call rates >98%. The threshold for successful genotyping was set to 95% for the FFPE TAB derived samples and using this cutoff, eight of the 12 samples passed initial genotyping quality control (QC). These eight samples had a ΔCT value <2 and a mean call rate of 97.4%. The remaining four samples with genotyping call rates <95% had ΔCT value >2 and a mean call rate of 89.2%. Linear regression analysis demonstrated a positive correlation between genotype missing rate and ΔCT values ($y = 0.034x - 0.0011$, $R^2 = 0.4308$, $p=0.02$) (**Figure 1**).

The GenCall (GC) scores and standard deviation of the logR ratios (log R Dev) as calculated by Genome Studio were also utilized as QC metrics. A GC score is a value between zero and one which reflects the reliability of each genotype call; scores >0.7 indicate high quality genotypes. The mean GC score of the eight FFPE samples which passed QC was 0.7370 while the peripheral blood derived samples had a comparable result of 0.7583. The log R Dev is based on normalized fluorescence intensity data and is a measure of reproducibility and signal-to-noise ratio. The average log R Dev of the eight FFPE samples was 0.50, which is consistent with values obtained using DNA from other sources such as blood or saliva (0.40) (Table 1).

All of the called genotypes for the eight FFPE samples which passed a stringent RT-PCR ΔCT <2 cutoff were compared to their matching peripheral blood DNA genotypes, the mean discordant heterozygote and mean discordant homozygote SNP calls were 0.0028 (range: 0.0007-0.0068) and 0.0003 (range: 0.0002-0.0006), respectively (Table 1).

In this study we directly compared genome-wide genotyping of FFPE and peripheral blood DNA samples obtained from the same patients. FFPE tissue specimens, such as TAB, hold an abundance of invaluable information for studies of human disease such as GCA. Regrettably, formalin fixation procedures and subsequent storage conditions result in nucleic acid degradation, DNA cross-linking and base modification[12]. Consequently, FFPE samples have previously yielded highly degraded DNA leading to sub-optimal performance in most microarray genotyping studies. Recent advances in DNA recovery and restoration methodologies specifically targeted towards FFPE specimens

have; however, enabled extraction of DNA of sufficient quality to be accurately genotyped using the microarray genotyping technology employed in GWAS.

Our results demonstrate that employing rigorous DNA QC thresholds, such as a ΔCT cutoff values of <2 for RT-PCR QC prior to DNA restoration, will dramatically improve genotyping quality and success rate. As expected, a slight increase in the genotype missing rate was observed in the FFPE derived samples; however, the number of discordant genotypes between the sample types is very low indicating that this methodology can produce robust and reproducible results. The rate of discordance in heterozygous genotype calls was slightly greater than homozygous calls. This finding is likely to be due to DNA damage such as deletions, introduced during the specimen fixation process, resulting in an under-representation of alternate alleles. Nonetheless, for the samples passing a stringent RT-PCR ΔCT <2 the level of discordance is consistent with that previously been described by Hong *et al.* who reported a total discordance rate of 0.0013 for intra-laboratory technical replicates genotyped on Illumina Omni 1M microarrays[13].

In summary, GCA is a complex disease and studies investigating its genetic architecture have been limited by their lack of statistical power, with the relatively late age of diagnosis contributing to the difficulties in recruiting large case cohorts. However, given that diagnosis is confirmed by TAB, archived FFPE histopathological specimens represent a valuable resource for large-scale molecular analysis. Adopting our QC FFPE-restoration thresholds will enable the large-scale ascertainment and utilization of samples from GCA patients for potential use in genome-wide association study, thereby circumventing the retrospective recruitment challenge for GCA. Ultimately this methodology is not only relevant to GCA but to other diseases requiring histological analysis and where recruitment of patients has proven difficult.

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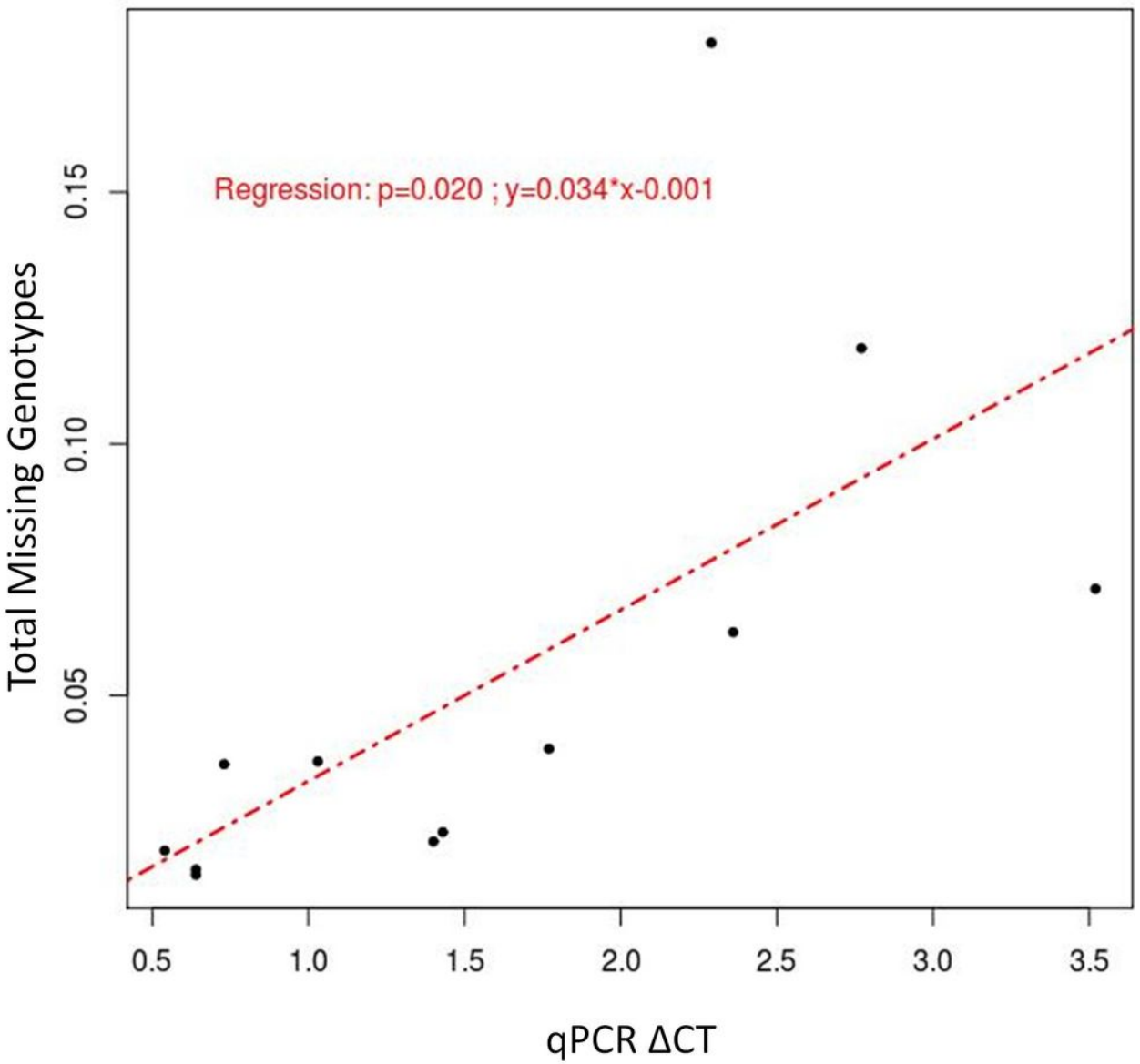
Table 1. Comparison of DNA extracted from archived FFPE temporal artery specimens and peripheral blood.

	FFPE derived DNA				Blood derived DNA			FFPE v Blood	
Patient	RT-PCR ΔCT	Total Missing Genotypes	Mean GC Score	LogR Dev	Total Missing Genotypes	Mean GC Score	LogR Dev	Discordant Heterozygous Genotypes	Discordant Homozygous Genotypes
1	0.54	0.0192	0.7441	0.4831	0.0097	0.7560	0.4356	0.0011	0.0003
2	0.64	0.0154	0.7486	0.4735	0.0086	0.7572	0.4262	0.0007	0.0002
3	0.64	0.0144	0.7508	0.4544	0.0071	0.7600	0.4075	0.0008	0.0002
4	0.73	0.0363	0.7244	0.5637	0.0121	0.7538	0.4000	0.0042	0.0005
5	1.03	0.0369	0.7237	0.5541	0.0078	0.7591	0.4122	0.0059	0.0006
6	1.40	0.0210	0.7431	0.4614	0.0101	0.7568	0.3851	0.0013	0.0002
7	1.43	0.0228	0.7397	0.5145	0.0086	0.7578	0.4308	0.0018	0.0003
8	1.77	0.0394	0.7215	0.5670	0.0126	0.7530	0.4072	0.0068	0.0005
9	2.29	0.1797	0.5838	0.5944	0.0066	0.7612	0.3938	0.1472	0.0049
10	2.36	0.0626	0.6992	0.5358	0.0058	0.7639	0.3128	0.0182	0.0007
11	2.77	0.1190	0.6401	0.6827	0.0084	0.7595	0.3653	0.0762	0.0033
12	3.52	0.0712	0.6834	0.6594	0.0059	0.7617	0.3854	0.0257	0.0015

Abbreviations: FFPE, formalin-fixed paraffin embedded; CT, cycle threshold; GC Score, GenCall score, LogR Dev, standard deviation of the logR ratio.

Figure 1. Correlation between qPCR ΔCT value and genotype missing rate


Regression: p=0.020 ; y=0.034*x-0.001
Total Missing Genotypes
0.05
0.10
0.15
0.5
1.0
1.5
2.0
2.5
3.0
3.5
qPCR ΔCT