EBioMedicine 4 (2016) 146–152

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Circulating Cell Free DNA in the Diagnosis of Trophoblastic Tumors

Mark R. Openshaw, Richard A. Harvey, Neil J. Sebire, Baljeet Kaur, Naveed Sarwar, Michael J. Seckl, Rosemary A. Fisher *

Trophoblastic Tumour Screening & Treatment Centre, Imperial College London, Charing Cross Campus, Fulham Palace Road, London W6 8RF, UK

ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form 21 December 2015
Accepted 26 December 2015
Available online 29 December 2015

Keywords:
Trophoblastic tumor
Hydatidiform mole
Cell free DNA
Circulating tumor DNA
Molecular genotyping
Liquid biopsy

ABSTRACT

Gestational trophoblastic neoplasia (GTN) represents a group of diseases characterized by production of human chorionic gonadotropin (hCG). Since non-gestational tumors may occasionally secrete hCG, histopathological diagnosis is important for appropriate clinical management. However, a histopathological diagnosis is not always available. We therefore investigated the feasibility of extracting cell free DNA (cfDNA) from the plasma of women with GTN for use as a "liquid biopsy" in patients without histopathological diagnosis. cfDNA was prepared from the plasma of 20 women with a diagnosis of GTN and five with hCG-secreting tumors of unknown origin. Genotyping of cfDNA from the patient, genomic DNA from her and her partner and DNA from the tumor tissue identified circulating tumor DNA (ctDNA) (from 9% to 53% of total cfDNA) in 12 of 20 patients with GTN. In one case without a tissue diagnosis, ctDNA enabled a diagnosis of GTN originating in a non-molar conception and in another a diagnosis of non-gestational tumor, based on the high degree of allelic instability and loss of heterozygosity in the ctDNA. In summary ctDNA can be detected in the plasma of women with GTN and can facilitate the diagnosis of both gestational and non-gestational trophoblastic tumors in cases without histopathological diagnosis.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Gestational trophoblastic neoplasia (GTN) is a spectrum of pregnancy related malignancies including invasive molar disease, choriocarcinoma and the much rarer placental site trophoblastic tumors (PSTT) and epithelioid trophoblastic tumors (ETT) (Hui et al., 2014). Prior to the development of cytotoxic chemotherapy these malignant conditions were invariably fatal. However, overall cure rates now exceed 98% due to the development of improved chemotherapeutic regimens and follow-up protocols (Seckl et al., 2010).

A hallmark of GTN is the production of human chorionic gonadotropin (hCG). Serum hCG levels aid rapid diagnosis and accurate disease monitoring (Seckl et al., 2013). However, hCG secretion alone is not always diagnostic of GTN as some non-gestational malignancies also secrete hCG (Iles et al., 2010). Women with GTN fall into two groups; (i) those who following evacuation of a molar pregnancy are treated with a clinical diagnosis of GTN based on rising serum hCG levels and (ii) those who present with an hCG-secreting tumor. For the second group histopathological examination of tissue is important for determining the correct diagnosis and when the diagnosis remains unclear molecular genotyping can play an important role (Fisher et al., 2007).

Most histological specimens of trophoblastic neoplasia are obtained via sampling of disease from the uterus. However GTN may be highly vascular and biopsy of tissue in the uterus or elsewhere may be deemed unsafe due to the risk of hemorrhage. Therefore some patients with metastatic disease, a raised serum hCG and characteristic history, such as a recent pregnancy, may be treated as a GTN without a histological diagnosis. This is because it is prudent to treat a highly curable disease, rather than risk morbidity and mortality via delay to achieve a histological diagnosis (Seckl et al., 2013). Patients who have GTN may therefore be left with uncertainty regarding their prognosis while patients with non-gestational tumors may be treated with inappropriate aggressive chemotherapy. For these women development of a blood based diagnostic test would be beneficial.

Circulating cell free DNA (cfDNA) has been investigated in patients with solid tumors and circulating tumor DNA (ctDNA) is reported to be detectable in a wide range of malignancies (Bettegowda et al., 2014). In solid tumors there is evidence that ctDNA is an effective biomarker at predicting relapse following surgery (Diehl et al., 2008) and progression during chemotherapy and targeted therapy (Diaz et al., 2012). In prenatal screening for aneuploidy, fetal cfDNA obtained from maternal plasma, has proven to be highly accurate with a detection rate for trisomy 21 of up to 100% (Norton et al., 2015). Since GTN is both a malignancy and pregnancy related we would predict cfDNA from trophoblastic cells to be present in the plasma of patients with these tumors. Due to the unique genetics of GTN i.e. the presence of

* Corresponding author.
E-mail address: r.fisher@imperial.ac.uk (R.A. Fisher).

http://dx.doi.org/10.1016/j.ebiom.2015.12.022
2352-3964/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

non-maternal DNA in the tumor, the DNA signature of these tumors may be easily detectable. cfDNA may therefore provide unique genetic information about a patients' disease hereto unavailable.

This report describes the feasibility of extracting cfDNA from the plasma of women with GTN, detection of ctDNA within these samples and utility of cfDNA to act as a "liquid biopsy" to enable the correct diagnosis for patients with hCG-secreting tumors without a tissue diagnosis.

2. Methods

2.1. Patients

Twenty-five patients were enrolled in the study. All patients were in their first week of admission to the Trophoblastic Screening and Treatment Centre, Charing Cross Hospital (CXH) to receive chemotherapy for confirmed or suspected GTN. Patients were split into two groups according to presentation.

2.2. Group 1: Women with Confirmed GTN

This group included 20 patients, 18 of whom were previously registered with the Trophoblastic Screening and Treatment Centre, following a histological diagnosis of molar pregnancy and subsequently admitted to CXH for chemotherapy following a diagnosis of invasive molar disease. Two further patients with no previous diagnosis of molar pregnancy presented with metastatic gestational choriocarcinoma, confirmed on biopsy, and were included following admission for curative chemotherapy.

2.3. Group 2: Women with hCG-secreting Tumors of Unknown Origin

This group included five patients who were referred to CXH with raised hCG levels and tumors at one or more sites but no histological diagnosis. All five patients were treated with a presumptive diagnosis of GTN. Two of these patients are described below.

2.4. Case CFD-023

A 47 year old female presented with vaginal bleeding. Obstetric history included two normal male pregnancies 15 and 13 years previously and a termination of pregnancy 11 years ago. Routine chest radiograph revealed cannon ball lung metastases. Her serum hCG at this point was 374,365 IU/L. Two days following admission the patient was transferred to CXH. CT staging revealed a pelvic mass and multiple pulmonary metastases. MRI head showed a 2 mm brain metastasis. No tissue was available for diagnosis and her FIGO score was 20. The patient was initially treated with low dose induction etoposide and cisplatin (EP) (Agarwal et al., 2014) weekly and once clinically stable was switched to EP, methotrexate and actinomycin-D for patients with central nervous system disease (EP-EMA(CNS)) plus intrathecal methotrexate (MTX) as she had ultra-high risk disease (Seckl et al., 2013).

2.5. Case CFD-008

A 33 year old female presented with abdominal pain and a positive pregnancy test. Her obstetric history was a normal pregnancy four years previously and a miscarriage three years ago. Her medical history was of a T3N1M0 gastric adenocarcinoma successfully treated 18 months previously with neoadjuvant epirubicin, cisplatin, and capecitabine chemotherapy. She was initially managed locally as a suspected ectopic pregnancy but her serum hCG climbed to >200,000 IU/L. A computed tomography scan showed widespread liver metastases and she was therefore transferred to CXH. Further imaging revealed no other abnormalities. No tissue was available for confirmation of the clinical diagnosis of GTN and her FIGO score was 21. Initial treatment was with EP-EMA(CNS) plus intrathecal MTX.

2.6. Preparation of Plasma

Plasma was separated from whole blood by centrifugation at 1900 $\times g$ for 10 min at 4 °C. The plasma layer was separated and a further centrifugation step at 16,000 $\times g$ for 10 min was included. Plasma was stored at −80 °C until analysis.

2.7. cfDNA Preparation

cfDNA was prepared from 3 mL plasma according to manufacturer's instructions using a QIAamp circulating nucleic acid kit (Qiagen, UK).

Table 1
Non-maternal cfDNA in women with gestational trophoblastic tumors.

Case	Serum hCG (IU/L)	Days since administration of chemotherapy	Total cfDNA from 3 mL plasma (ng)	Estimated % of cfDNA that is ctDNA based on genotyping	Estimated ctDNA (ng)	Diagnosis based on ctDNA	Genotype of antecedent molar pregnancy
Post-mole trophoblastic tumors							
CFD-001	10,097	1	9.8	0	–		
CFD-002	12,946	6	17.4	0	–		
CFD-003	37,923	6	14.4	0	–		
CFD-004	35,095	3	6.0	18	1.08	GTN; post-CHM	Androgenetic, monospermic
CFD-005	1497	3	3.2	0	–		
CFD-007	448,650	6	8.6	42	3.61	GTN; post-CHM	Androgenetic, monospermic
CFD-009	14,884	2	24.2	0	–		
CFD-010	16,326	3	17.1	10	1.71	GTN; post-CHM	Not available
CFD-011	24,622	3	4.4	16	0.70	GTN; post-CHM	Androgenetic, monospermic
CFD-012	8308	2	5.9	0	–		
CFD-013	133,018	5	22.1	42	9.28	GTN; post-CHM	Not available
CFD-015	20,237	6	8.5	0	–		
CFD-016	30,227	2	16.2	12	1.94	GTN; post-CHM	Androgenetic, dispermic
CFD-018	238,703	7	4.6	13	0.60	GTN; post-CHM	Androgenetic, monospermic
CFD-019	53,046	0	5.4	0	–		
CFD-022	47,472	1	4.3	26	1.11	GTN; post-CHM	Androgenetic, monospermic
CFD-024	150,101	5	14.6	19	2.78	GTN; post-CHM	Androgenetic, monospermic
CFD-025	169,442	5	10.8	11	1.19	GTN; post-CHM	Androgenetic, dispermic
Post-term choriocarcinoma							
CFD-027	66,861	5	9.3	9	0.84	GTN	NA
CFD-031	700,855	1	23.7	53	12.60	GTN; post-male	NA

hCG, human chorionic gonadotropin; cfDNA, cell free DNA; ctDNA, circulating tumor DNA; CHM, complete hydatidiform mole; GTN, gestational trophoblastic neoplasia; NA, not applicable.

Table 2
Non-maternal cfDNA in women with hCG-secreting tumors of unknown origin.

Case	Serum hCG (IU/L)	Reproductive history	Total cfDNA from 3 mL plasma (ng)	Estimated % of cfDNA that is ctDNA based on genotyping	Estimated ctDNA (ng)	Diagnosis based on ctDNA
CFD-008	239,171	FTNDF, miscarriage	123.0	65	79.95	Non-gestational tumor
CFD-014	5254	FTNDM	14.1	–	–	–
CFD-017	6	No known pregnancy	28.8	–	–	–
CFD-023	372,117	FTNDM, FTNDM, TOP	118.0	44	51.92	Post-term tumor (female)
CFD-026	725	No known pregnancy	15.7	–	–	–

FTNDF, full term normal delivery of female; FTNDM, full term normal delivery of male; TOP, termination of pregnancy; cfDNA cell free DNA; ctDNA circulating tumor DNA.

cfDNA was quantified using a Picogreen dsDNA quantitation kit (Life Technologies, UK).

2.8. Genomic DNA Preparation

DNA was prepared from 200 μL of patient's blood, and where possible their partner, using a QIAamp DNA mini kit (Qiagen, UK) as per the manufacturers protocol.

2.9. DNA Preparation from Tissue

Trophoblastic tissue and adjacent maternal tissue were dissected independently from 3 to 5 unstained 5 μm sections of formalin-fixed paraffin-embedded (FFPE) tissue, with reference to a Hematoxylin and Eosin stained section. DNA was prepared using a QIAmp DNA FFPE Tissue Kit (Qiagen, UK) according to the manufacturer's protocol and the DNA stored at -20 °C until analysis.

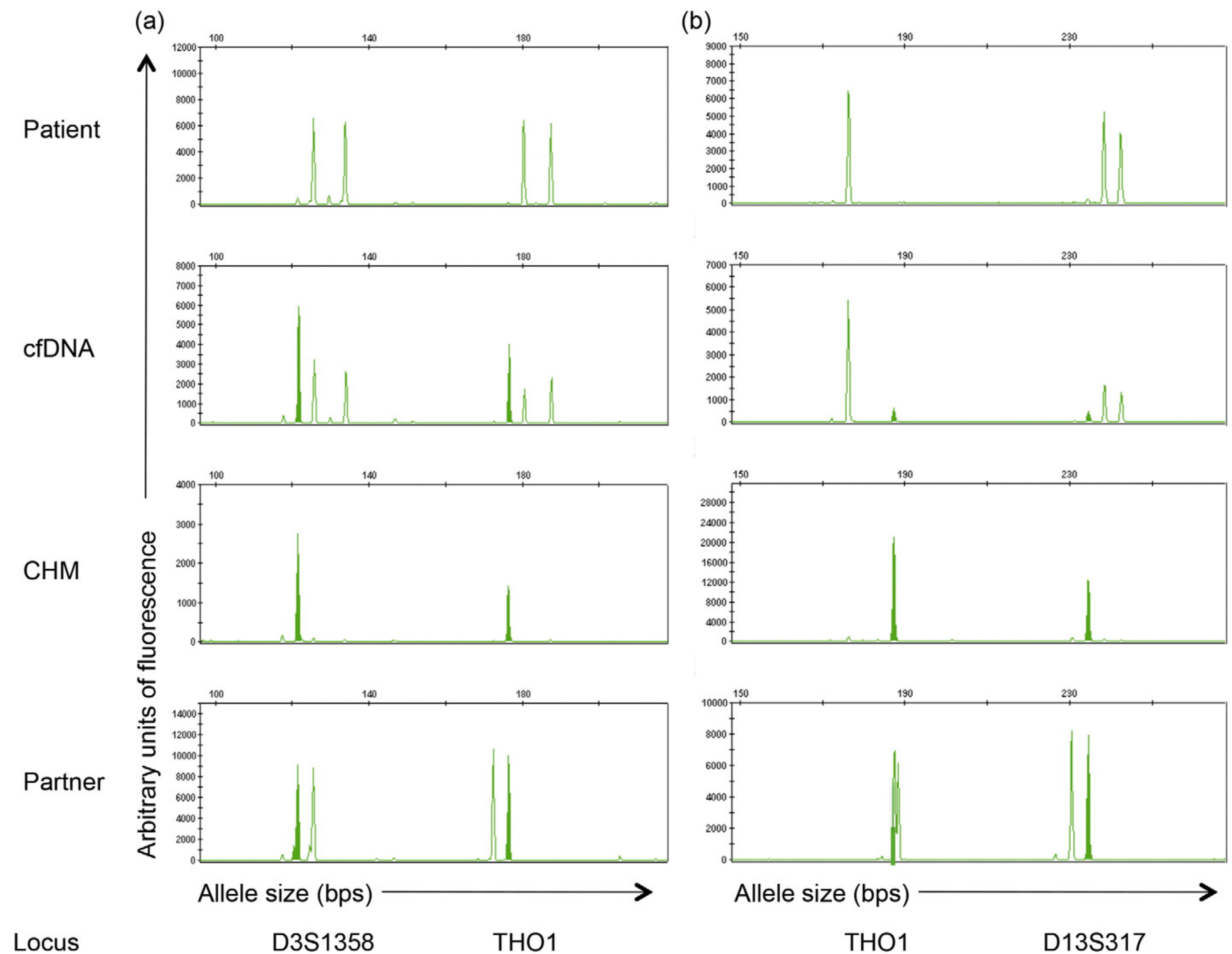

Fig. 1. Partial genotype of cfDNA, DNA from the patient, her partner, and the CHM in two cases of post-mole GTN. (a) Case CFD-007 showing a single (solid) peak, found in the paternal sample and the antecedent CHM, at the D3S1358 locus in addition to two maternally derived (open) peaks. The relative height of the paternal peak (3626 units) and the maternal peaks (2656 and 1745 units) provides an estimate of the ctDNA of 45% (3626/8027). Relative peak heights of 2065, 1400 and 1129 units at the THO locus also give an estimate of ctDNA of 45% (2065/4595). (b) Case CFD-016 showing a single paternally derived (solid) peak in addition to a single, or two, maternal (open) peak(s) at the THO1 and D13S317 loci respectively. The relative heights of the peaks at the THO1 and D13S317 locus give an estimated percentage of ctDNA of 11% (650/6060) and 15% (509/3595) respectively in this case. Similar results were obtained when area under the peak, rather than peak height, was used to estimate ctDNA.

2.10. DNA Genotyping

1 μL DNA was amplified with primers for 15 short tandem repeat (STR) loci on 13 chromosomes, plus the amelogenin locus, using an AmpFlSTR Identifiler Plus kit (Applied Biosystems, Warrington, UK). PCR products were resolved by capillary electrophoresis using an ABI 3100 Genetic Analyzer and genotypes determined using GeneMapper version 4.0 software (Applied Biosystems, Warrington, UK).

Where non-maternal alleles were present in the amplified cfDNA and were consistent with the presence of ctDNA originating from a molar pregnancy, the percentage of DNA attributable to the molar tissue was estimated by calculating the height of the peak(s) for the paternal allele(s) as a proportion of the sum of the allele heights for all alleles at that locus. The proportion of ctDNA was calculated for three informative loci and the average recorded. Where a paternal allele was present and disproportionate heights of the maternal alleles suggested the ctDNA originated in a non-molar pregnancy, the percentage of ctDNA was estimated from the relative height of the paternal allele to the sum of the paternal allele plus the smaller maternal allele, representing cfDNA of maternal origin. Again the average for three informative loci was recorded.

2.11. Statistics

The yield of ctDNA and serum hCG levels were compared using Spearman's correlation analysis with a p value <0.05 being considered significant.

2.12. Ethics

The project was approved (project no. R14021) by the Tissue Management Committee of the Imperial College Healthcare NHS Trust Research Tissue Bank which is approved by NRES to provide deemed ethics for projects associated with the Research Tissue Bank. All patients in the study and their partners consented to providing additional blood samples.

2.13. Role of the Funding Source

Funding and resources were provided by the Trophoblastic Screening and Treatment Centre, Charing Cross Hospital, London, UK, the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre and the Imperial Experimental Cancer Medicine Centre. The funding sources played no role in the design, completion or decision to publish this article.

3. Results

3.1. Extraction of cfDNA from Plasma

cfDNA was detectable in all 25 cases. Total cfDNA extracted from 3 mL of plasma ranged from 3.2 to 123.0 ng (Tables 1, 2).

3.2. cfDNA in Women with Confirmed GTN

cfDNA was detectable in all 20 cases with GTN. In these 20 cases the total cfDNA extracted from 3 mL plasma ranged from 3.2 to 24.2 ng (Table 1). Of the 18 cases of known post-mole GTN, genotyping of the cfDNA in ten (56%) revealed alleles of non-maternal origin, consistent with ctDNA originating in CHM. In eight cases genotyping of the antecedent molar pregnancy confirmed this as the origin of the ctDNA (Table 1; Fig. 1). The percentage of detectable ctDNA ranged from 10% to 42% of the total cfDNA (Table 1) while total ctDNA ranged from 0.6 to 9.28 ng.

ctDNA was detectable in both patients with choriocarcinoma diagnosed on routine histopathology (Table 1). In case CFD-031 an estimated 53% of the cfDNA was tumor in origin (Fig. 2). A greater proportion of cfDNA for one of the two maternal alleles at informative loci, and the presence of DNA from a Y chromosome demonstrated that this tumor had arisen in a previous male pregnancy. This was confirmed by genotyping of DNA from the tumor tissue (Fig. 2). In case CFD-027 the proportion of cfDNA attributable to the tumor was small (9%). Although non-maternal alleles could be identified in the cfDNA, the high level of cfDNA from the patient's own cells obscured any potential maternal contribution to the tumor genotype and so, although clearly gestational, it was not possible to determine whether the causative pregnancy had been a normal conception or an androgenetic CHM.

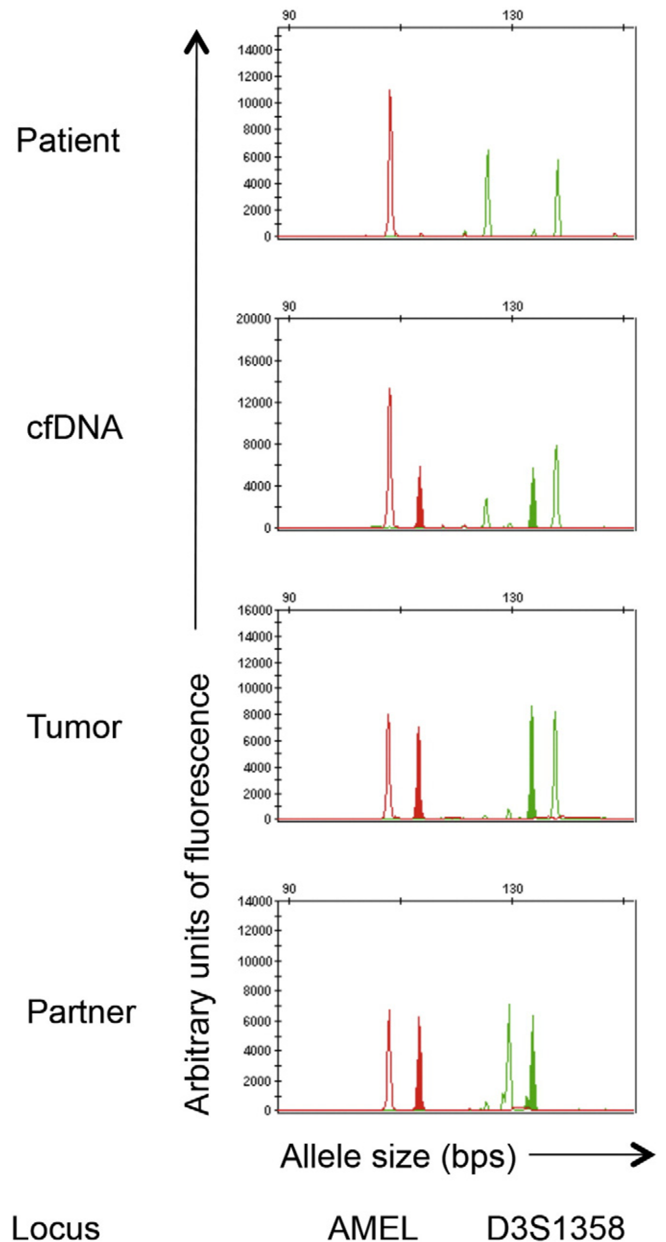

Fig. 2. Partial genotype of cfDNA, DNA from the patient, her partner and the tumor tissue in case CFD-031. The presence of a Y chromosome, represented by the solid peak for the amelogenin (AMEL) locus, confirms a paternal contribution to the cfDNA. At the D3S1358 loci a single paternally derived allele (solid peak) is present together with two maternally derived alleles (open peaks), the disproportionate areas under these two peaks indicating that one maternally derived peak comprises DNA from both ctDNA and maternal cfDNA. The higher maternal peak represents ctDNA and cfDNA of maternal origin in the same ratio as the solid peak representing only ctDNA (5735 units) and the lower maternal peak representing only cfDNA of maternal origin (2919 units) providing an estimate of percentage ctDNA of 66% (5735/8674).

3.3. ctDNA and hCG Levels

The presence of ctDNA in women with GTN was found to be associated with serum hCG levels. No ctDNA was detected in cases where serum hCG levels were 14,884 IU/L or below; ctDNA was detectable, but not consistently, in serum hCG levels between 16,326–53,046 IU/L while ctDNA was consistently detected at serum hCG levels of 66,861 IU/L and above (Table 1). For women with GTN Spearman's rank correlation coefficient showed a significant correlation between serum hCG concentrations and ctDNA (rho = 0.69; 95% CI 0.35 to 0.87; p = 0.001).

3.4. cfDNA in Women with hCG-secreting Tumors of Unknown Origin

In three women with tumors of unknown origin, the genotype of the cfDNA was the same as that of the patient. In two further cases non-maternal cfDNA was present enabling a diagnosis to be made (Table 2) and these are discussed in further detail.

3.5. Case CFD-023

Analysis of cfDNA extracted from plasma taken before chemotherapy, detected paternal alleles in the cfDNA, in addition to both maternal alleles, thereby confirming the presence of a gestational tumor (Fig. 3). For D21S11 and other loci, not shown, the two maternal alleles were present at different heights suggesting one was derived from maternal cfDNA and the other made up of maternal cfDNA and ctDNA. The genotype of the tumor is therefore consistent with an origin in a pregnancy from a normal, non-molar conception with both a maternal and paternal contribution to the genome. Genotyping at the AMEL locus showed all the cfDNA to be from the X-chromosome (Fig. 3) and therefore unlikely to have originated in either of her full term male pregnancies. The tumor was most likely to have originated in her terminated pregnancy, which was presumably female. This patient has ongoing response to primary chemotherapy.

3.6. Case CFD-008

Analysis of cfDNA extracted post chemotherapy revealed no alleles in the cfDNA that were not found in the patient indicating an absence of any contribution to the tumor genome from her partner. However, the genotype of the cfDNA was very different to that of the patient showing a high degree of allelic instability and some loss of heterozygosity when compared to the patient's genotype (Fig. 4), suggesting that a high proportion of the cfDNA was in fact ctDNA. The absence of alleles other than those in the patient was consistent with a tumor of non-gestational origin. There was an initial complete radiological response to chemotherapy but her hCG began to rise 5 months later. Genotyping of tissue from a subsequent liver biopsy showed a very similar pattern to that of the cfDNA (Fig. 4) confirming that the cfDNA contained ctDNA that was non-gestational in origin. An additional allele seen in the later biopsy, at locus D13S317, suggests further instability and some tumor heterogeneity between the ctDNA and biopsied tissue. Following a histological diagnosis of non-gestational disease the patient switched to palliative treatment and died one month later.

4. Discussion

Analysis of ctDNA is currently being introduced as a non-invasive method for detecting and monitoring the progress of a number of cancers (Bettegowda et al., 2014; Crowley et al., 2013). In this study we have demonstrated that ctDNA can be detected in the plasma of women with trophoblastic tumors and can facilitate diagnosis.

Screening using fetal cfDNA from maternal blood samples is increasingly used for prenatal diagnosis (Bianchi, 2012; Nicolaides et al., 2014). While cfDNA is usually cleared rapidly after delivery (Lo et al., 1999) we speculated that cfDNA would persist in women with invasive molar

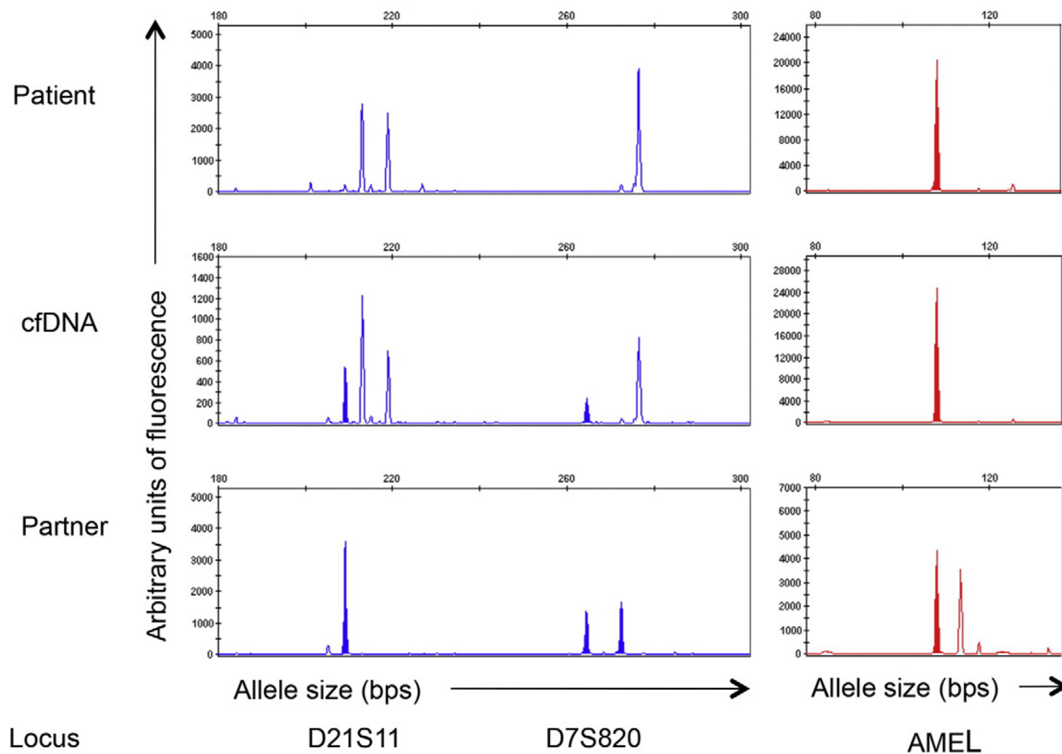

Fig. 3. Partial genotype of cfDNA, DNA from the patient, and from her partner in case CFD-023. A paternal contribution to the cfDNA is represented by a single allele (solid peak) of 540 and 242 units for loci D21S11 and D7S820 respectively. The disproportionate heights of the two maternal alleles at the D21S11 locus indicate that one peak represents both ctDNA and maternal cfDNA. The percentage cfDNA was calculated as above giving an estimate for percentage ctDNA of 44% (540/1237) in this case. Genotyping of the amelogenin locus (AMEL) showed that all the cfDNA has an XX gender and does not contain a Y chromosome, indicating that the tumor had originated in a female pregnancy.

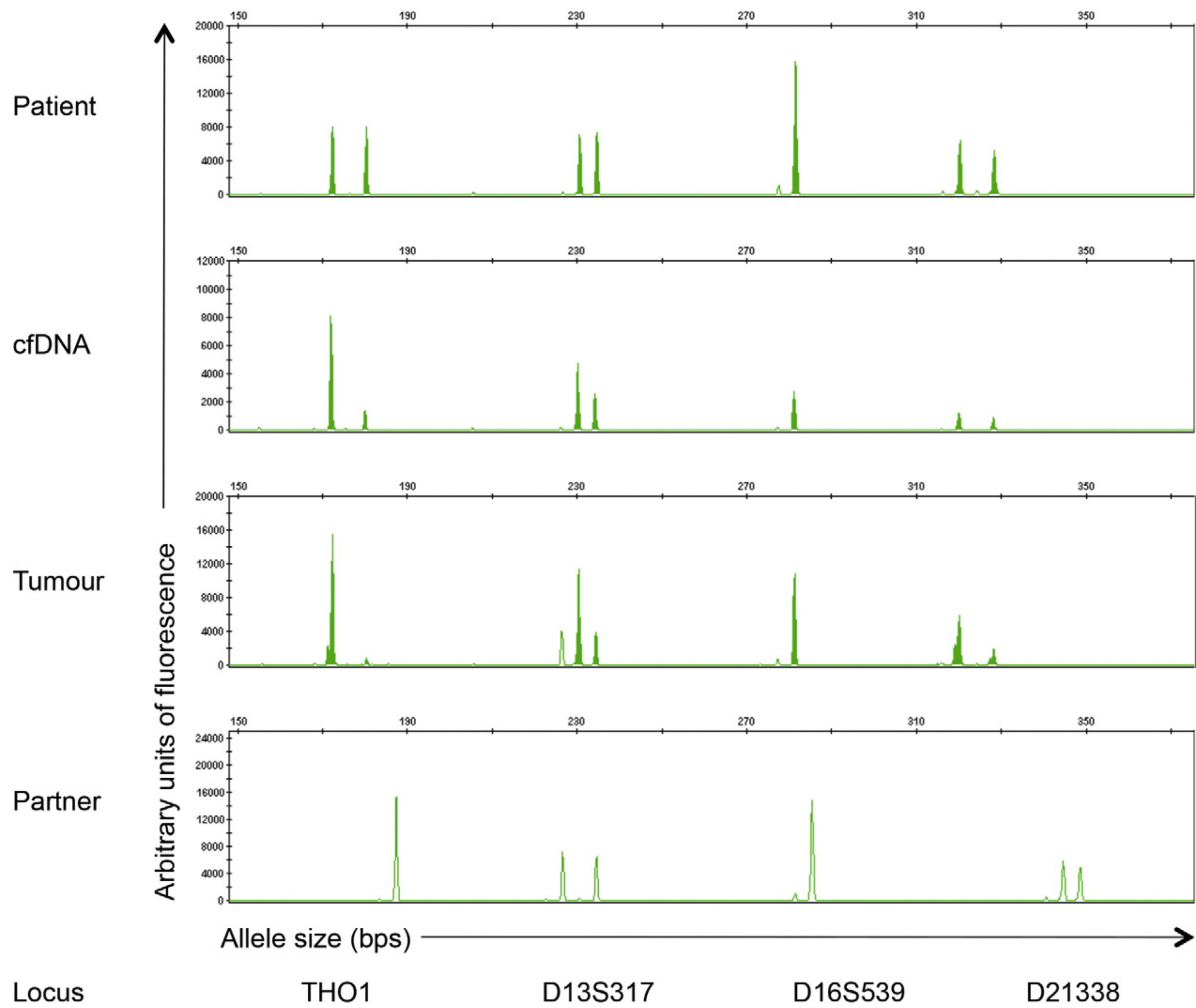

Fig. 4. Partial genotype of cfDNA, DNA from the patient, her partner and a subsequent DNA sample from a liver metastasis in case CFD-008. While all alleles in the cfDNA (solid peaks) are present in the patient's DNA the proportion of each allele is very different suggesting that, where there are two alleles, the lower peak in each case represents cfDNA from the patient while the higher peak represents mostly ctDNA. The contribution of maternal cfDNA to the higher peak would be expected to be similar to that of the lower peak. ctDNA is therefore represented by the height of the higher peak less the lower peak for informative markers, (8058–1356 units), 71% of the total cfDNA, for the THO1 locus.

disease, as trophoblastic tissue continues to grow following termination of the pregnancy in these cases. This study has shown that for the majority of women who develop trophoblastic tumors, there is sufficient ctDNA in patients' blood to be detected by molecular genotyping, a technique routinely used in clinical diagnosis of trophoblastic disease (Fisher et al., 2007, 2014).

Both the total cfDNA extracted from 3 mL of plasma, 3.2–24.2 ng, and the proportion of total cfDNA that derived from the molar pregnancy, 0–42%, were highly variable. In other tumor types the level of detectable ctDNA has been reported to relate to the extent of disease (Diehl et al., 2008; Dawson et al., 2013; Kim et al., 2014). Since serum hCG levels reflect tumor burden in GTN (Seckl et al., 2010, 2013), the yield of ctDNA might be expected to parallel serum hCG levels. The yield of ctDNA reflected tumor burden in that ctDNA was detectable in all patients with a serum hCG of 66,861 IU/L and above but undetectable where serum hCG was 14,884 IU/L or below. While Spearman's correlation coefficient for serum hCG levels and yields of ctDNA was significant in women with GTN overall, there were a group of women with serum levels between 16,326 IU/L and 53,046 IU/L where detection of ctDNA was variable, suggesting that the release of ctDNA from tumors following chemotherapy may reflect other aspects of tumor biology and not simply tumor burden. Further studies analyzing ctDNA levels pre- and post-chemotherapy at specific time points are needed to investigate the exact relationship between serum hCG levels and ctDNA yields.

With the rare exception of a small number of PSTT and ETT, all GTN produce hCG. While hCG production is a characteristic of all GTN, a number of other tumor subtypes have been shown to secrete hCG ranging from 4% in prostate cancer to as high as 76% of bladder tumors (Iles et al., 2010). For most of these tumors other clinicopathological features are likely to lead to a diagnosis but for women of reproductive age with raised serum hCG and no pathological diagnosis, the differential diagnosis may be between a GTN and a non-gestational, hCG-secreting malignancy (Fisher et al., 2007). In two of five cases of women with hCG-secreting tumors of unknown origin in the present series, genotyping of cfDNA was helpful in establishing a diagnosis. In one patient the presence of ctDNA enabled a diagnosis of a GTN originating in a female pregnancy. In the other genotyping of ctDNA showed gross microsatellite instability consistent with ctDNA from a non-gestational tumor. The same microsatellite instability was demonstrated in the liver metastasis that was later biopsied indicating the unequivocal origin of the cfDNA from the tumor. These two cases demonstrate the utility of cfDNA to provide a 'liquid biopsy' to aid diagnosis when histology is unavailable and that genotyping can potentially diagnose both gestational and non-gestational trophoblastic tumors.

In three cases of tumor of unknown origin it was not possible to make a specific genetic diagnosis as all cfDNA had an identical genotype to the patient. It is possible that ctDNA was present and that the tumors were non-gestational but as the ctDNA showed no instability at the loci

analyzed the ctDNA was indistinguishable from that of the patient. This was confirmed in one case (CFD 017) in which genotyping of the tumor tissue, from a subsequent biopsy, showed the tumor to be non-gestational with a genotype identical to the patient for those loci analyzed. Alternatively ctDNA may have been below the level of detection in these cases as all three patients had very low levels of serum hCG. While genotyping using short tandem repeat polymorphisms is relatively straightforward other techniques such as droplet digital PCR (Hudecova, 2015), in combination with single nucleotide polymorphisms (Matsuda and Honda, 2015), might provide a more sensitive approach for identifying ctDNA in women with low serum hCG. Conversely the absence of paternal alleles in a patient with a high level of serum hCG might suggest a non-gestational tumor. Further studies are needed to more accurately determine the current sensitivity of genotyping assays for this type of analysis.

While genotyping is useful in the differential diagnosis of gestational and non-gestational trophoblastic tumors, it does not distinguish choriocarcinoma from the much rarer PSTT or ETT, which have a poorer prognosis and may require different therapeutic approaches (Lurain, 2011). cfDNA from patients with PSTT or ETT could provide ctDNA which together with next generation sequencing or microarray analysis might enable identification of specific genetic alterations associated with these tumor types. Such biomarkers would potentially provide a means of monitoring response in these rare tumors for which serum hCG levels may be less informative than for the more common GTN, choriocarcinoma (Moutte et al., 2013).

Identification of biomarkers that could identify women in whom molar pregnancy will not resolve spontaneously following termination or biomarkers that predict resistant disease are important objectives in GTN management. cfDNA has enabled tracking of specific known mutations and identification of de-novo mutations in other tumor types (Frenel et al., 2015; Patel and Tsui, 2015; Thierry et al., 2014). ctDNA from women with GTN together with advancements in modern technology, that enable analysis of very small DNA samples, offers the potential to provide answers to these questions.

This study has shown that ctDNA can be extracted from women with GTN and can act as a liquid biopsy enabling diagnosis when a histopathological diagnosis is not available. While these observations need to be validated in larger studies, the ability to isolate ctDNA from GTN offers the potential for improved diagnosis, more personalized monitoring of disease, and a resource to investigate and expand our knowledge of this rare group of tumors.

Declaration of Interests

Authors have declared no conflict of interest.

Authors Contributions

Mark Openshaw: Literature search, patient recruitment, consent, data collection, data analysis & interpretation, writing manuscript.

Richard A Harvey: Data analysis & interpretation, statistics, writing manuscript.

Neil J Sebire: Histopathology, data collection, data analysis, writing manuscript.

Baljeet Kaur: Histopathology, data collection, data analysis, writing manuscript.

Naveed Sarwar: Patient recruitment, data interpretation, writing manuscript.

Michael J Seckl: Patient recruitment, data interpretation, writing manuscript.

Rosemary A Fisher: Study design, literature search, data collection, data analysis & interpretation, figures, writing manuscript.

Acknowledgments

We are grateful to the patients who consented to take part in this study. Tissue samples were provided by the Imperial College Healthcare NHS Trust Tissue Bank funded by the NIHR Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.12.022.

References

Agarwal, R., Alifrangis, C., Everard, J., et al., 2014. Management and survival of patients with FIGO high-risk gestational trophoblastic neoplasia: the U.K. experience, 1995–2010. J. Reprod. Med. 59, 7–12.

Bettegowda, C., Sausen, M., Leary, R.J., et al., 2014. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci. Transl. Med. **6**, 224ra24.

Bianchi, D.W., 2012. Prenatal diagnostics: fetal genes in mother's Blood. Nature 487, 304–305.

Crowley, E., Di Nicolantonio, F., Loupakis, F., Bardelli, A., 2013. Liquid biopsy: monitoring cancer-genetics in the blood. Nat. Rev. Clin. Oncol. 10, 472–484.

Dawson, S.J., Rosenfeld, N., Caldas, C., 2013. Circulating tumor DNA to monitor metastatic breast cancer. N. Engl. J. Med. 369, 93–94.

Diaz Jr., L.A., Williams, R.T., Wu, J., et al., 2012. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 486, 537–540.

Diehl, F., Schmidt, K., Choti, M.A., et al., 2008. Circulating mutant DNA to assess tumor dynamics. Nat. Med. 14, 985–990.

Fisher, R.A., Savage, P.M., MacDermott, C., et al., 2007. The impact of molecular genetic diagnosis on the management of women with hCG-producing malignancies. Gynecol. Oncol. 107, 413–419.

Fisher, R.A., Tommasi, A., Short, D., Kaur, B., Seckl, M.J., Sebire, N.J., 2014. Clinical utility of selective molecular genotyping for diagnosis of partial hydatidiform mole; a retrospective study from a regional trophoblastic disease unit. J. Clin. Pathol. 67, 980–984.

Frenel, J.S., Carreira, S., Goodall, J., et al., 2015. Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecularly targeted drug administration. Clin. Cancer Res. 21, 4586–4596.

Hudecova, I., 2015. Digital PCR analysis of circulating nucleic acids. Clin. Biochem. 48, 948–956.

Hui, P., Baergen, R., Cheung, A.N.Y., et al., 2014. Gestational Tophoblastic Neoplasms. WHO Classification of Tumours of Female Reproductive Organs. International Agency for Research on Cancer, Lyon, p. 158.

Iles, R.K., Delves, P.J., Butler, S.A., 2010. Does hCG or hCGβ play a role in cancer cell biology? Mol. Cell. Endocrinol. 329, 62–70 (Mol Cellular Endocrinol 2007; **260–262**: 264–70).

Kim, K., Shin, D.G., Park, M.K., et al., 2014. Circulating cell-free DNA as a promising biomarker in patients with gastric cancer: diagnostic validity and significant reduction of cfDNA after surgical resection. Ann. Surg. Treat. Res. 86, 136–142.

Lo, Y.M., Zhang, J., Leung, T.N., Lau, T.K., Chang, A.M., Hjelm, N.M., 1999. Rapid clearance of fetal DNA from maternal plasma. Am. J. Hum. Genet. 64, 218–224.

Lurain, J.R., 2011. Gestational trophoblastic disease II: classification and management of gestational trophoblastic neoplasia. Am. J. Obstet. Gynecol. 204, 11–18.

Matsuda, K., Honda, T., 2015. High-speed droplet-allele-specific polymerase chain reaction for genotyping of single nucleotide polymorphisms. Methods Mol. Biol. 1310, 29–35.

Moutte, A., Doret, M., Hajri, T., et al., 2013. Placental site and epithelioid trophoblastic tumours: diagnostic pitfalls. Gynecol. Oncol. 128, 568–572.

Nicolaides, K.H., Syngelaki, A., del Mar, G.M., Quezada, M.S., Zinevich, Y., 2014. Prenatal detection of fetal triploidy from cell-free DNA testing in maternal blood. Fetal Diagn. Ther. 35, 212–217.

Norton, M.E., Jacobsson, B., Swamy, G.K., et al., 2015. Cell-free DNA analysis for noninvasive examination of trisomy. N. Engl. J. Med. 372, 1589–1597.

Patel, K.M., Tsui, D.W., 2015. The translational potential of circulating tumour DNA in oncology. Clin. Biochem. 48, 957–961.

Seckl, M.J., Sebire, N.J., Berkowitz, R.S., 2010. Gestational trophoblastic disease. Lancet 376, 717–729.

Seckl, M.J., Sebire, N.J., Fisher, R.A., Golfier, F., Massuger, L., Sessa, C., 2013. Gestational trophoblastic disease: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann. Oncol. **24** (Suppl. 6), vi39–vi50.

Thierry, A.R., Mouliere, F., El Messaoudi, S., et al., 2014. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat. Med. 20, 430–435.